Aus der Klinik für angeborene Herzfehler und Kinderkardiologie
des Deutschen Herzzentrums Berlin

DISSERTATION

Hypothermie-induzierte zelluläre und molekulare Mechanismen in ischämisch geschädigten Zellen

zur Erlangung des akademischen Grades
Doctor rerum medicinalium (Dr. rer. medic.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

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Datum der Promotion: 18.12.2020

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AIF	Apoptosis inducing factor
AP-1	Activator protein 1
ATP	Adenosintriphosphat
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
cDNA	Complementary DNA
CIRBP	Cold-inducible RNA-binding protein
CsA	Cyclosporin A
Cyto C	Cytochrom C
DAMP	Damage-associated molecular pattern
DIV	Days *in vitro*
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HMGB1	High mobility group box 1
Hsp70	Heat shock protein 70
Iba1	Ionized calcium-binding adapter molecule 1
IL-6	Interleukin-6
IL-1β	Interleukin-1 beta
iNOS	Inducible nitric oxide synthase
I/R	Ischämie/Reperfusion
LC3	Microtubule-associated protein 1A/1B-light chain 3
LDH	Laktatdehydrogenase
MAPK	Mitogen-activated protein kinase
MCP1	Monocyte chemotactic protein 1
mPTP	Mitochondriale Permeabilitäts-Transitions-Pore
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
OGD	Oxygen-Glucose-Deprivation
OGD/R	OGD und Reperfusion
OHSC	Organotypische hippocampale Schnittkulturen
PLL	Poly-L-Lysin
PI	Propidiumiodid
RBM3	RNA-binding motif protein 3
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
TH	Therapeutische Hypothermie
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TGF-β1	Transforming growth factor-beta1
TTM	Targeted temperature management
ZNS	Zentrales Nervensystem

1 Zusammenfassung

1.1 Abstrakt – Deutsch

Hypoxisch-ischämische Ereignisse, die beispielsweise durch Herzkreislaufstillstand oder Schlaganfall verursacht werden, führen zu schwerer Zellschädigung. Das Absenken der Körpertemperatur stellt dabei eine effektive Methode zum Schutz vor Ischämie- und Reperfusionsschädigung im Gehirn und Herzen dar. Die genauen protektiven Mechanismen sind jedoch noch nicht vollständig verstanden. Intrazerebral kann Hypothermie einen Ischämie-induzierten Zelltod reduzieren und die dadurch hervorgerufene Neuroinflammation beeinflussen. Ähnliche neuroprotektive Eigenschaften werden durch die Applikation des Immunsuppressors Cyclosporin A (CsA) diskutiert.

In der ersten Studie wurde der Einfluss der Hypothermie, der Behandlung mit CsA sowie die Kombination beider Interventionen nach einstündiger Sauerstoff-Glukose-Deprivation, gefolgt von 24-stündiger Reperfusion (OGD/R) in murinen BV-2 Mikroglia, primären Neuronen und organotypischen hippocampalen Schnittkulturen (OHSC) untersucht. Die postischämische Kühlung auf 33,5 °C reduzierte den Zelluntergang von OGD-geschädigten OHSC und konnte die Aktivierung der BV-2 Mikroglia mindern. Die Behandlung mit 10 µM CsA reduzierte die Expression von IL-1β und Iba1 in BV-2 Mikroglia, hatte jedoch einen toxischen Effekt auf OGD-geschädigte primäre Neurone und OHSC. Eine Kombination beider Interventionen hatte keinen zusätzlichen Effekt auf BV-2 Mikroglia und primäre Neurone, führte allerdings in OGD/R-geschädigten OHSC zu einer gesteigerten Inflammation gemessen durch die Induktion der TNF-α, MCP1, IL-6 und Iba1 Genexpression.

Angelehnt an das Protokoll für Kühlung nach perinataler Asphyxie wurde in der zweiten Studie der Effekt einer bis zu 72-stündigen Kühlung nach einer 24-stündigen, schweren (0,2 % O_2) oder moderaten (8 % O_2) Hypoxie untersucht. Schwere Hypoxie führte zum Zelluntergang in der humanen neuronalen Zelllinie SK-N-SH, welcher durch die Kühlung auf 33,5 °C reduziert wurde. Gleichzeitig wurde die Expression des Kälteschockprotein RBM3 unter Kühlung erhöht. Somit stellt die RBM3-Expression einen möglichen, durch Hypothermie vermittelten Schutz bei Hypoxie-geschädigten neuronalen Zellen dar.

In der dritten Studie wurde am Modell eines Myokardinfarkts der Effekt einer intraischämischen Kühlung in OGD/R-geschädigten murinen HL-1 Kardiomyozyten untersucht. Die Zellen wurden innerhalb der sechsstündigen OGD-Phase und in der Reperfusion für insgesamt 24 Stunden auf 33,5 °C gekühlt. Hypothermie erhielt die Integrität der mitochondrialen Membran und verhinderte dabei die Ausschüttung von AIF und Cyto C, induzierte das antiapoptotische Hsp70, erhöhte das Bcl-2/Bax Verhältnis und die LC3-II Expression. Der Einsatz früher Hypothermie führte somit zum Schutz der Herzmuskelzellen vor intrinsischer Apoptose und aktivierte zudem protektive Autophagiemechanismen.

Zusammenfassend wurden durch die Studien weitere zugrunde liegende, protektive Mechanismen der Hypothermie bei Ischämie- und Reperfusionsschädigung aufzeigt. Zudem wurde in der Arbeit erstmalig beschrieben, dass die Kombinationstherapie aus Hypothermie und CsA-Behandlung in komplexen Gewebekulturen nicht neuroprotektiv wirkt, sondern eine Neuroinflammation induziert.

1.2 Abstract – Englisch

Hypoxic-ischemic events occur as a result of cardiac arrest or stoke can lead to severe cell damage. Cell death in the brain and heart after ischemia and reperfusion damage is most effectively reduced by hypothermia, but the underlying protective mechanisms are not yet fully understood. However, the neuroinflammatory response induced by ischemic cell death is a major secondary injury mechanism that has been shown to be attenuated by hypothermia. Similar neuroprotective properties of the immunosuppressor cyclosporin A (CsA) are also discussed.

In the first study, we investigated the effects of hypothermia, treatment with CsA, and a combined treatment of both interventions on oxygen-glucose deprived and reperfused (OGD/R) murine BV-2 microglia, primary neurons, and organotypic hippocampal slice cultures (OHSC). Post-ischemic cooling to 33.5 °C decreased OGD-induced cell death in OHSC, and reduced activation of BV-2 microglia. Treatment with 10 µM CsA reduced expression of IL-1β and Iba1 in BV-2 microglia, but was toxic in OGD–damaged neurons and OHSC. Combined treatment of post-ischemic cooling and CsA during OGD/R had no further effect on BV-2 microglia and primary neurons, but increased inflammation in OHSC by induced expression of TNF-α, MCP1, IL-6, and Iba1.

According to clinical cooling guidelines after perinatal asphyxia, in the second study we investigated the neuroprotective effects of 72 hours long hypothermia and RBM3 expression by mild (8 % O_2) and severe (0.2 % O_2) hypoxia-induced injury in human SK-N-SH neuronal cells. Severe hypoxia followed by reoxygenation lead to cell death, which was reduced by cooling. Simultaneously, protein expression of RBM3 was upregulated by cooling. As a result, hypothermia induced RBM3 expression might be an important factor in rescuing hypoxic damaged neuronal cells.

In the third study, we investigated the effects of intra-ischemic cooling on OGD/R damaged murine HL-1 cardiomyocytes as a model of cardiac arrest. Hypothermic treatment preserved mitochondrial membrane integrity and reduced releases of AIF and cytochrom C. Simultaneously, anti-apoptotic Hsp70, Bcl2/Bax-ratio, and autophagic LC3-II expressions were induced by cooling. Early application of hypothermia thereby protects the cardiomyocytes by inhibiting intrinsic apoptosis and activating protective autophagy mechanisms.

In summary, we showed hypothermia-mediated mechanisms to protect cells from ischemia and reperfusion induced damage. Additionally, we described for the first time that combined hypothermia and CsA treatment in the complex slice cultures is not neuroprotective, but induces neuroinflammation that could result in increased neurotoxicity.

1.3 Einführung

Hypoxisch-ischämische Ereignisse verursacht durch beispielsweise Herzkreislaufstillstand oder Schlaganfall führen zu schwerer Zellschädigung und sind der Hauptgrund für Morbidität und Mortalität weltweit [1]. Die Schädigung des Gewebes erfolgt zunächst durch die Unterbrechung der Versorgung während der Ischämiephase und anschließend durch die erneute Durchblutung und schnelle Wiederbereitstellung von Sauerstoff im Gewebe, der Reperfusion. Auf zellulärer Ebene beeinträchtigt die Schädigung durch Ischämie/Reperfusion (I/R) die gesamte Zellphysiologie. In der Phase der Ischämie kommt es durch einen gesteigerten anaeroben Metabolismus zu einer Senkung des pH-Werts, einer ATP-Abnahme, dem Verlust des Membranpotenzials und zu Ionenkanal-Dysfunktionen. Ein gesteigerter Einstrom von Kalzium-Ionen in die Zelle führt zu einer zellulären Schwellung sowie zur Aktivierung von Proteasen und Endonukleasen, wodurch Apoptose, Autophagie und nekrotischer Zelltod ausgelöst werden können [2, 3]. Der Schaden in der Reperfusion wird ausgelöst durch die schnelle Wiederversorgung mit Sauerstoff, wodurch es zur Bildung von reaktiven Sauerstoffspezies, der Öffnung der mitochondrialen Permeabilitäts-Transition-Pore (mPTP) und der Einleitung von mitochondrialer Dysfunktion kommt und somit die Induktion von nekrotischem oder apoptotischem Zelltod zur Folge hat [4].

Neben dem Zelluntergang durch I/R-induzierte Schädigung kann es im ischämischen Gewebe zu einer sterilen Inflammation kommen. Im Gegensatz zu einer durch Pathogene induzierten Inflammation, wird die sterile Inflammation durch Zellschädigung ohne Einwirkung von Pathogenen hervorgerufen. Schädigungsassoziierte molekulare Muster (*damage-associated molecular pattern*, DAMP), die von nekrotischen Zellen ausschüttet werden, binden an *pattern-recognition-receptors* (z.B. an *toll-like* Rezeptoren, TLR) benachbarter immunkompetenter Zellen und können dort eine Inflammationsreaktion auslösen und dadurch die Pathogenese von Gewebeschädigung verstärken [5-8].

Der therapeutische Einsatz der Kühlung nach I/R-induzierter Schädigung hat sich klinisch vor allem zum Schutz des Gehirns bewährt, da es das Organ mit der geringsten Ischämietoleranz darstellt. Mechanistisch konnte gezeigt werden, dass die Kühlung komplexe Abläufe der Zelle moduliert und I/R-induzierten Schädigungen wie Ionen-Dysbalance, mitochondrialer Dysfunktion, oxidativem Stress, Inflammation, Exzitotoxizität und Apoptose entgegenwirken kann [9]. Im Rahmen der Kühlung ist die gezielte Temperaturkontrolle von großer Bedeutung. In einer Vielzahl klinischer Studien konnte gezeigt werden, dass das Herabsetzen der Körpertemperatur auf 32-34 °C nach einer perinatalen Asphyxie und hypoxisch-ischämischer Enzephalopathie bei Neugeborenen die Mortalität mindert und das neurologische Outcome der Kinder deutlich verbessert [10, 11]. Weiterhin kamen verschiedene Studien zu dem Ergebnis, dass die Kühlung auf 32-36 °C nach einem Herzkreislaufstillstand außerhalb des Krankenhauses die Überlebenschancen steigert und die neurologische Langzeitprognose verbessert, sodass die therapeutische Hypothermie unter anderem in die amerikanischen und europäischen medizinischen Leitlinien aufgenommen wurde [12-14]. Ebenso wurde gezeigt, dass Proteine der Kälteschockfamilie durch die Kühlung induziert werden und protektive Eigenschaften aufweisen [15, 16].

Weitere klinische Studien zeigten, dass neben der Kühlung die gezielte Temperaturkontrolle (*targeted temperature management*, TTM) von großer Bedeutung ist. So konnte in einer klinischen, randomisierten, multizentrischen Studie, bei der die Patienten nach einer Reanimation auf 33 °C gekühlt wurden, kein Vorteil gegenüber der ebenfalls gekühlten Kontrollgruppe bei 36 °C gezeigt werden [17]. Daten aus präklinischen Nagetierstudien unterstützen zudem einen Einsatz von Kühlung als neuroprotektive Strategie nach Schlaganfall [18, 19]. Der klinische Einsatz von Hypothermie nach einem akuten ischämischen Schlaganfall ist derzeit jedoch noch umstritten. So wurde in einer klinischen Studie die Gefahr einer Pneumonie und eine Zunahme der Mortalität im Vergleich zur ungekühlten Gruppe festgestellt [20].

Der Wirkstoff Cyclosporin A (CsA) wird derzeit als potentiell neuroprotektive Substanz erforscht und diskutiert. Klinisch wird CsA primär in der Transplantationsmedizin zur Suppression von T-Zellen verwendet. Intrazellulär bindet CsA an Cyclophilin A und inhibiert die Proteinphosphatase Calcineurin, welche die Immunantwort über die Aktivierung von Transkriptionsfaktoren wie dem *nuclear factor of activated T cells* (NFAT) induziert [21, 22]. Zusätzlich bindet CsA an Cyclophilin D und verhindert die Bildung einer mPTP, wodurch eine mitochondriale Dysfunktion und folglich Apoptose aufgehalten werden kann [23, 24].

Zielsetzungen

Ziel der ersten Studie (Wowro et al. [25]) war es, den Einfluss der Kühlung und der CsA-Behandlung auf Vitalität und Neuroinflammation von I/R-geschädigten *in vitro*-Monokulturen von Neuronen und immunkompetenten Mikroglia zu untersuchen. Zusätzlich wurde das Protokoll auf komplexe organotypische hippocampale *ex vivo*-Schnittkulturen (OHSC) übertragen, da sich diese auf Grund von erhaltenen Gewebestrukturen und Zell-Zell-Verbindungen besser zur Untersuchung neuroprotektiver Strategien eignen [26]. Ein besonderer Schwerpunkt wurde dabei auf die durch simulierte I/R-induzierte sterile Inflammation und der damit einhergehenden Neuroinflammation gelegt. Der Einfluss der beiden putativ neuroprotektiven Behandlungen auf eine Neuroinflammation wurde hier nach aktuellem Wissen erstmalig beschrieben. In der zweiten Studie (Rosenthal et al. [27]) wurde die neuroprotektive Wirkung der Kühlung auf hypoxisch geschädigte neuronale Zellen und die Expressionskinetik des Kälteschockproteins *RNA binding motif protein 3* (RBM3) untersucht. In der dritten Studie (Krech et al. [28]) wurde der Einfluss der intraischämischen Kühlung auf I/R-geschädigten Kardiomyozyten untersucht. Ein Fokus wurde hierbei auf den Einfluss der Hypothermie auf die mitochondriale Dysfunktion und Apoptose gelegt.

1.4 Material und Methodik

Eine ausführliche Beschreibung der verwendeten Materialien und Methoden sind den jeweiligen Publikationen zu entnehmen. Eine Zusammenfassung der verwendeten Methoden wird im Folgenden dargestellt.

1.4.1 Publikation 1: Die Kombination von Hypothermie und Cyclosporin A reduziert die Aktivierung von BV-2 Mikroglia, induziert jedoch eine Inflammation in Ischämie/Reperfusion-geschädigten hippocampalen Schnittkulturen [25]

Es wurden murine BV-2 Mikroglia, primäre murine Neurone aus dem Kortex und dem Hippocampus (15. Embryonaltag) sowie murine organotypische hippocampale Schnittkulturen (OHSC, postnataler Tag 1-3) für die Experimente verwendet. Alle Zellmodelle wurden zunächst einer einstündigen, simulierten Ischämie in glukose- und serumfreiem Medium hypoxischen Bedingungen (0,2 % O_2) bei 37 °C ausgesetzt. Anschließend erfolgte eine 24-stündige, simulierte Reperfusionsphase unter Wiederbereitstellung von Glukose, Serum und Sauerstoff (21 %) bei 37 °C oder 33,5 °C. Die Behandlung mit 1 oder 10 µM CsA erfolgte eine Stunde vor der Ischämie und wurde über die gesamte Dauer des Versuches aufrechterhalten (Abbildung 1).

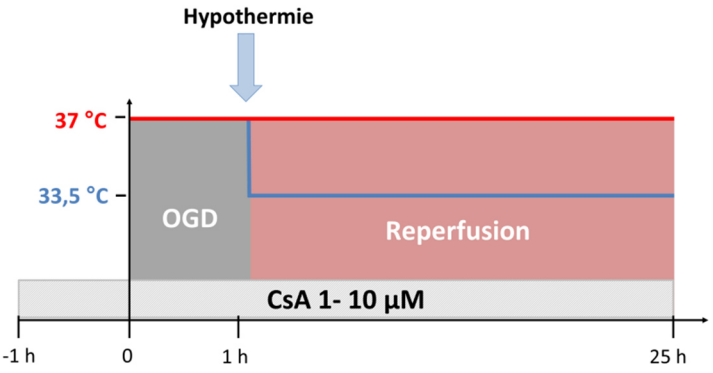

Abbildung 1. Zeit-Temperatur-Protokoll. Cyclosporin (CsA) wurde eine Stunde vor der einstündigen, simulierten Ischämie (*Oxygen-Glucose-Deprivation,* OGD) und der folgenden, 24-stündigen Reperfusion appliziert und über die Dauer des Versuchs belassen. Die Kühlung auf 33,5 °C erfolgte in der Reperfusionsphase. Zusätzlich zur Behandlungsgruppe bei 37 °C wurden Kontrollgruppen ohne OGD-Behandlung mitgeführt (modifiziert nach Wowro et al.[25]).

Zur Bestimmung einer zytotoxischen Wirkung wurde je nach Modell die Laktatdehydrogenase (LDH) im Zellkulturüberstand bestimmt oder der Zelltod mittels Propidiumiodid (PI)-Färbung dargestellt. Von geschädigten Zellen ausgeschüttete DAMPs, wie *heat shock protein 70* (Hsp70), *high mobility group box 1* (HMGB1) und *cold-inducible RNA-binding protein* (CIRBP), wurden aus dem Zellkulturüberstand durch Trichloressigsäure-Fällung isoliert und mittels Western Blot-Analyse quantifiziert. Die Isolation der RNA erfolgte zum Ende des Experiments nach 24-stündiger Reperfusion, gefolgt von der reversen Transkription zu cDNA. Die Genexpression von *interleukin-1 beta* (IL-1β), *tumor necrosis factor-alpha* (TNF-α), *Interleukin-6* (IL-6), *monocyte chemotactic protein 1* (MCP1), *inducible nitric oxide synthase*

(iNOS), *ionized calcium-binding adapter molecule 1* (Iba1) und *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH) wurde mit Hilfe des TaqMan® Genexpressionsassay an einem Real-Time PCR-System analysiert. Alle Ergebnisse wurden aus mindestens drei unabhängigen Versuchen gewonnen.

1.4.2 Publikation 2: Neuroprotektion durch die Expression von RBM3 in humanen SK-N-SH Neuronen wird durch Hypothermie, aber nicht Hypoxie reguliert [27]

Die humane Neuronenzelllinie SK-N-SH wurde für 24 Stunden einer moderaten (8 % O_2) oder einer schweren Hypoxie (0,2 % O_2) bei 37 °C ausgesetzt. Anschließend wurden die Zellen für weitere 24 bis 72 h unter atmosphärischer O_2-Konzentration (21 %) unter normothermen (37 °C) oder hypothermen (33,5 °C) Bedingungen kultiviert. Die Zytotoxizität wurde durch die Ausschüttung von LDH und der Neuronenspezifischen Endolase (NSE) im Zellkulturüberstand bestimmt. Strukturelle Veränderungen wurden mittels Immunfluoreszenzfärbungen mit βIII-Tubulin dargestellt. Durch die quantitative Bestimmung von ATP wurde die metabolische Aktivität der Zellen bestimmt. Zudem wurde die Expression des Kälteschockproteins RBM3 mittels quantitativer RT-PCR und durch eine Western Blot-Analyse bestimmt. Alle Ergebnisse wurden aus mindestens drei unabhängigen Versuchen gewonnen.

1.4.3 Publikation 3: Moderate therapeutische Hypothermie induziert protektive Mechanismen in Sauerstoff- Glukose-deprivierten und Reperfusion-geschädigten Kardiomyozyten [28]

Die murine Kardiomyozyten Zelllinie (HL-1) wurde einer sechsstündigen, simulierten Ischämie in glukose- und serumfreiem Medium hypoxischen Bedingungen (0,2 % O_2) ausgesetzt. Anschließend erfolgte eine 21-stündige, simulierte Reperfusionsphase unter Wiederbereitstellung von Glukose, Serum und Sauerstoff (21 %). Drei Stunden nach Beginn der Ischämie wurde eine Gruppe auf 33,5 °C gekühlt und für 24 h bei dieser Temperatur kultiviert. Die ungekühlte Kontrollgruppe wurde bei 37 °C kultiviert. Die mitochondriale Integrität wurde anhand der Ausschüttung vom a*poptose inducing faktor* (AIF) und *cytochrom C* (Cyto C) aus dem Mitochondrium ins Zytosol mittels Western Blot-Analyse bestimmt. Zum Nachweis einer mitochondrialen Dysfunktion wurden die Mitochondrien durch Mitotracker Red™ angefärbt und die Verteilung von Cyto C mittels Immunfluoreszenzfärbung visualisiert. Die Expression der antiapoptotischen Proteine *B-cell lymphoma 2* (Bcl-2) und Hsp70, des proapoptotischen *Bcl-2-associated X protein* (Bax) sowie des Markers für Autophagie *microtubule-associated protein 1A/1B-light chain 3* (LC3-II) wurde mittels Western Blot analysiert. Alle Ergebnisse wurden aus mindestens vier unabhängigen Versuchen gewonnen.

1.5 Ergebnisse

1.5.1 Publikation 1: Die Kombination von Hypothermie und Cyclosporin A reduziert die Aktivierung von BV-2 Mikroglia, induziert jedoch eine Inflammation in Ischämie/Reperfusion-geschädigten hippocampalen Schnittkulturen [25]

Neben der therapeutischen Kühlung wird der immunsuppressive Wirkstoff CsA als potenziell neuroprotektive Substanz diskutiert. In dieser Studie wurde die Wirkung von CsA während einer simulierten I/R *in vitro* in murinen primären Neuronenkulturen, in BV-2 Mikroglia und in *ex vivo* murinen OHSC untersucht. Zusätzlich wurde der Einfluss der post-ischämischen Kühlung auf 33,5 °C untersucht.

Der einstündige Entzug von Sauerstoff und Glukose (*Oxygen-Glucose-Deprivation*, OGD) führte in keinem der untersuchten Zellmodelle zum Anstieg des Zelltodmarkers LDH im Zellkulturüberstand. Bei gleichzeitiger Behandlung mit 10 µM CsA wurde signifikant mehr LDH von den primären Neuronen unter OGD und Normoxie und bei den OHSC unter OGD-Bedingungen freigesetzt, jedoch nicht in den BV-2 Mikroglia (Abbildung 2 A/B/C).

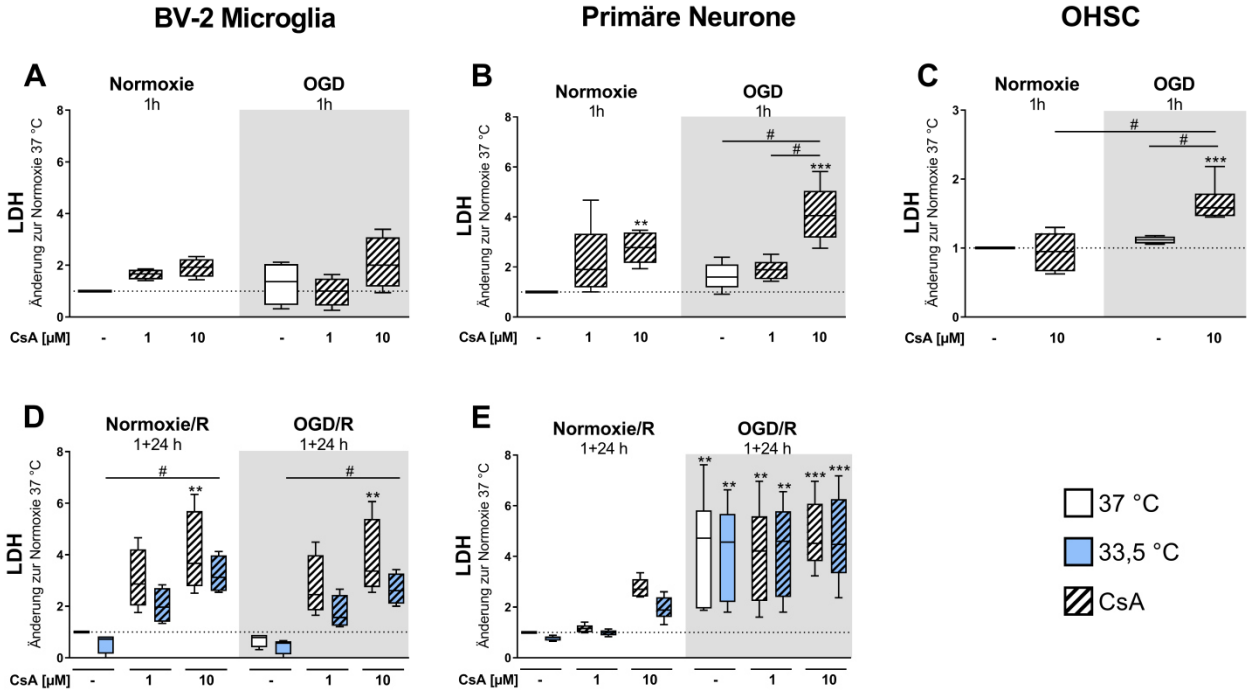

Abbildung 2. Laktatdehydrogenase (LDH)-Nachweis im Zellkulturüberstand von (A) BV-2 Mikroglia, (B) primären Neuronen und (C) organotypischen hippocampalen Schnittkulturen (OHSC) unter Kontrollbedingungen (Normoxie, 21 % O_2) und nach einstündiger, simulierter Ischämie (*Oxygen-Glucose-Deprivation*, OGD) bei 37 °C, sowie nach 24-stündiger, simulierter Reperfusion unter Kontrollbedingungen (Normoxie/R) und OGD/R (1 h OGD+ 24 h Reperfusion) in (D) BV-2 Mikroglia und (E) primären Neuronen. Alle Cyclosporin A (CsA)-Gruppen wurden für eine Stunde vorbehandelt. Daten aus n= 3-6 unabhängigen Experimenten sind als Box-Whiskers-Plots dargestellt. Signifikante Unterschiede wurden mittels einseitiger ANOVA mit anschließendem Tukey *post-hoc*-Test berechnet und sind mit *p<0,05, **p<0,01, ***p<0,001 (im Vergleich zu Normoxie, 37 °C) und mit #p<0,05 (im Gruppenvergleich) gekennzeichnet (modifiziert nach Wowro et al. [25]).

Anschließend an die OGD-Phase erfolgte die simulierte Reperfusion bei 37 °C oder 33,5 °C. In den BV-2 Mikroglia war nach OGD/R kein Anstieg an LDH verglichen mit der Kontrollgruppe (Normoxie/R) messbar. Bei gleichzeitiger Behandlung mit CsA kam es jedoch zu einem signifikanten, konzentrationsabhängigen Anstieg des LDH in den Normoxie/R- sowie den OGD/R-Gruppen unter 37 °C. Die Kühlung auf 33,5 °C verminderte den Zelltod tendenziell (Abbildung 2 D). Im Gegensatz dazu wurde signifikant mehr LDH nach der OGD/R in den primären Neuronen ausgeschüttet. Weder die Behandlung mit CsA noch die Kühlung auf 33,5 °C hatten einen zusätzlichen Einfluss auf den Zelltod (Abbildung 2 E). Aufgrund hoher Serumkonzentrationen im OHSC-Vollmedium war keine LDH-Bestimmung möglich. Alternativ zeigte die Färbung für den Zelltod einen Anstieg PI-positiver Zellen durch OGD/R, welche durch Kühlung auf 33,5 °C tendenziell vermindert wurde. Bei gleichzeitiger CsA-Behandlung war jedoch kein Unterschied zu den OGD/R-Gruppen zu erkennen (Abbildung 3).

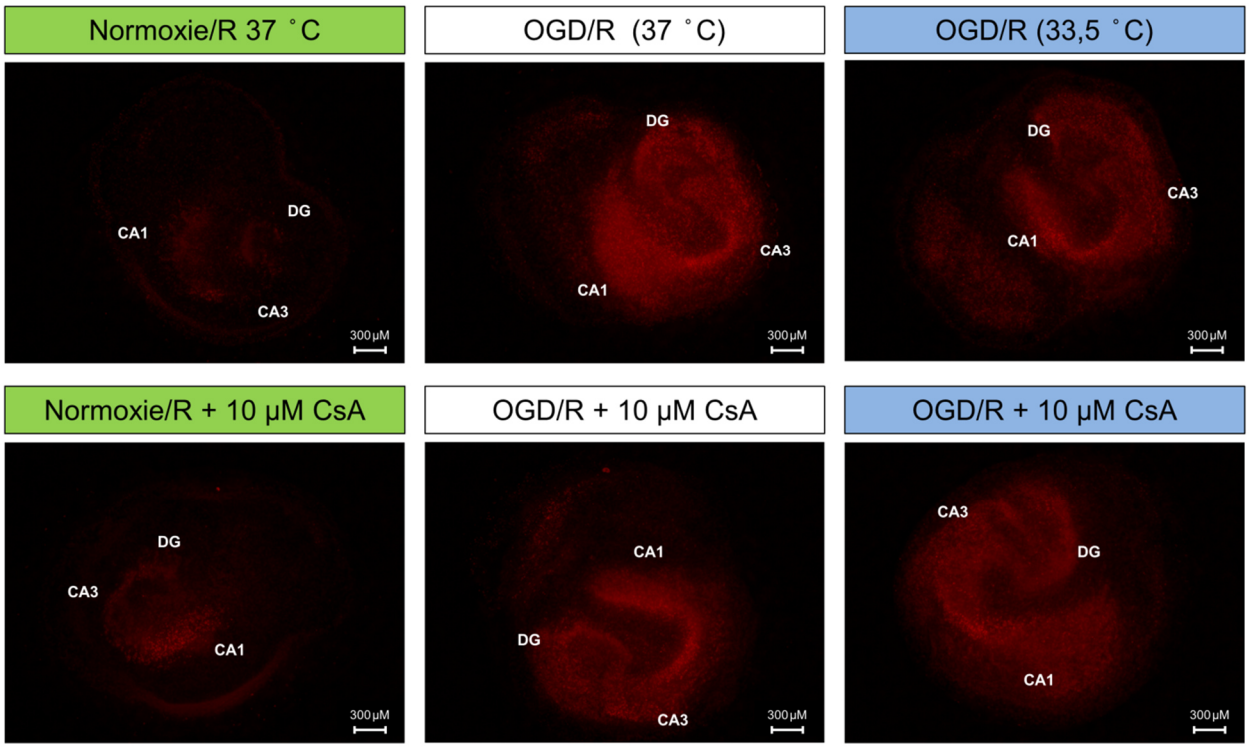

Abbildung 3. Propidiumiodid (PI)-Färbung organotypischer hippocampaler Schnittkulturen (OHSC). OHSC wurden unter Kontrollbedingungen (Normoxie/R 1 h+ 24 h) oder simulierter Ischämie+ Reperfusion (1 h OGD+ 24 h Reperfusion; OGD/R) bei 37 °C oder 33,5 °C kultiviert und anschließend mit PI gefärbt. Die Cyclosporin A (CsA)-Gruppen wurden für eine Stunde vorbehandelt. DG: Gyrus Dentatus, CA1|3: cornu ammonis1|3. Exemplarische, fluoreszenzmikroskopische Aufnahmen unter 40-facher Vergrößerung (modifiziert nach Wowro et al.[25]).

Nekrotischer Zelltod geht einher mit der Dissoziation der Zellmembran und Ausschüttung löslicher intrazellulärer Bestandteile. Daher wurden im nächsten Schritt extrazelluläre Proteine im Zellkulturüberstand nach der OGD-Phase isoliert und die Menge an Inflammation auslösenden DAMPs mittels Western Blot-Analyse quantifiziert. Korrelierend mit den LDH-Daten konnte kein signifikanter Anstieg an extrazellulären Hsp70 und HMGB1 in BV-2 Mikroglia detektiert werden (Abbildung 4 A/B). Jedoch war ein signifikanter Anstieg von extrazellulären Hsp70 und HMGB1 in primären Neuronen nach

OGD +10 µM CsA im Vergleich zur Normoxie-Kontrollgruppe sowie zu den OGD-Gruppen nachweisbar (Abbildung 4 C/D/E). Die untersuchten extrazellulären Proteine in den OHSC sowie CIRBP in den BV-2 Mikroglia waren mittels Western Blot-Analysen nicht nachweisbar.

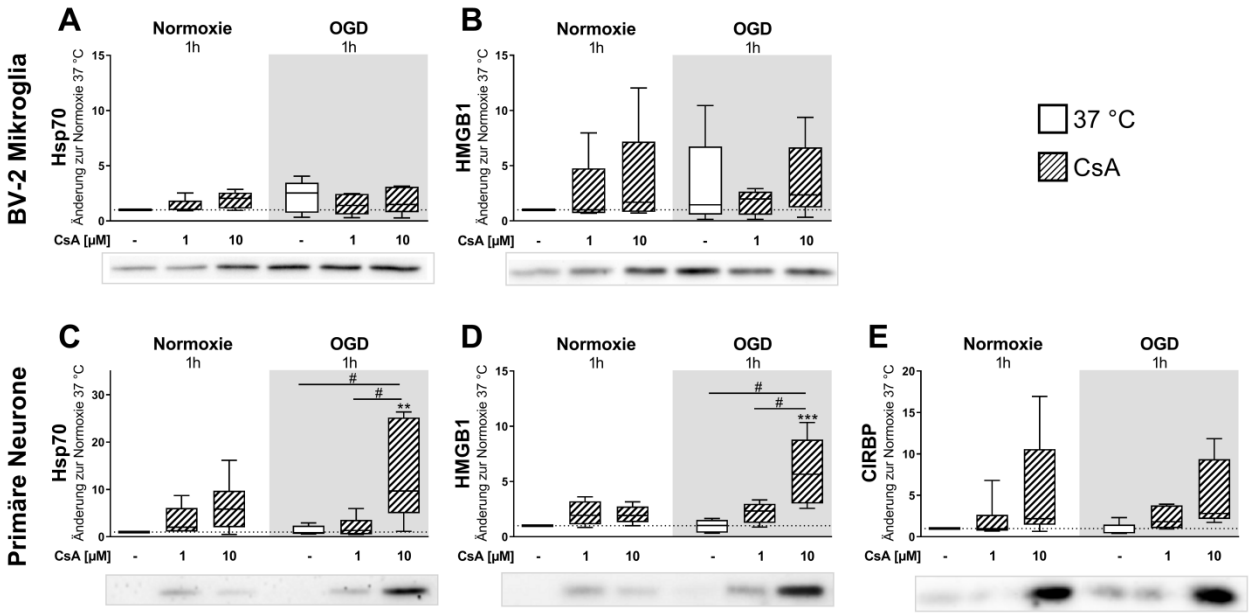

Abbildung 4. Western Blot-Analysen von *heat shock protein 70* (Hsp70), *high mobility group box 1* (HMGB1) und *cold-inducible RNA-binding protein* (CIRBP) aus dem Zellkulturüberstand von (A-B) BV-2 Mikroglia und (C-E) primären Neuronen unter Kontrollbedingungen (Normoxie, 21 % O_2) und nach einstündiger, simulierter Ischämie (*Oxygen-Glucose-Deprivation*, OGD) bei 37 °C. Alle Cyclosporin A (CsA)-Gruppen wurden für eine Stunde vorbehandelt. Daten aus n= 5-6 unabhängigen Experimenten sind als Box-Whiskers-Plots dargestellt. Signifikante Unterschiede wurden mittels einseitiger ANOVA mit anschließendem Tukey *post-hoc*-Test berechnet und sind mit **p<0,01, ***p<0,001 (im Vergleich zu Normoxie, 37 °C) und mit #p<0,05 (im Gruppenvergleich) gekennzeichnet (modifiziert nach Wowro et al.[25]).

Von nekrotischen Zellen ausgeschüttete DAMPs können eine Inflammationsreaktion in benachbarten Zellen auslösen. Daher wurde die Genexpression von Inflammation- und Aktivierungs-assoziierten Proteinen in den immunkompetenten BV-2 Mikroglia, den OHSC, aber auch in den primären Neuronen nach der Reperfusionsphase untersucht.

In den BV-2 Mikroglia war die Genexpression von TNF-α in der Normoxie/R- und OGD/R-Gruppe durch Kühlung signifikant induziert, bei gleichzeitiger Behandlung mit 10 µM CsA jedoch wieder vermindert. In den OHSC hingegen wurde durch OGD/R+CsA signifikant mehr TNF-α im Vergleich zur Normoxie/R-Gruppe, jedoch nicht in den Normoxie/R+CsA exprimiert (Abbildung 5 A/B). Die Expression des proinflammatorischen IL-1β und des Mikroglia-Aktivierungsmarker Iba1 wurde in den BV-2 Mikroglia durch die Behandlung mit CsA in der Normoxie/R- und OGD/R-Phase bei 37 °C signifikant reduziert. Während durch Kühlung die IL-1β-Expression unverändert blieb, war die Iba1-Expression tendenziell erhöht (Abbildung 5 C/E). In den OHSC konnte eine leichte Suppression von IL-1β nach Normoxie/R+CsA gegenüber der Normoxie/R-Kontrollgruppe gemessen werden, die jedoch durch OGD/R aufgehoben wurde. Die Expression von Iba1 wurde in den OHSC durch OGD/R unabhängig von CsA induziert und durch Kühlung tendenziell reduziert (Abbildung 5 D/F).

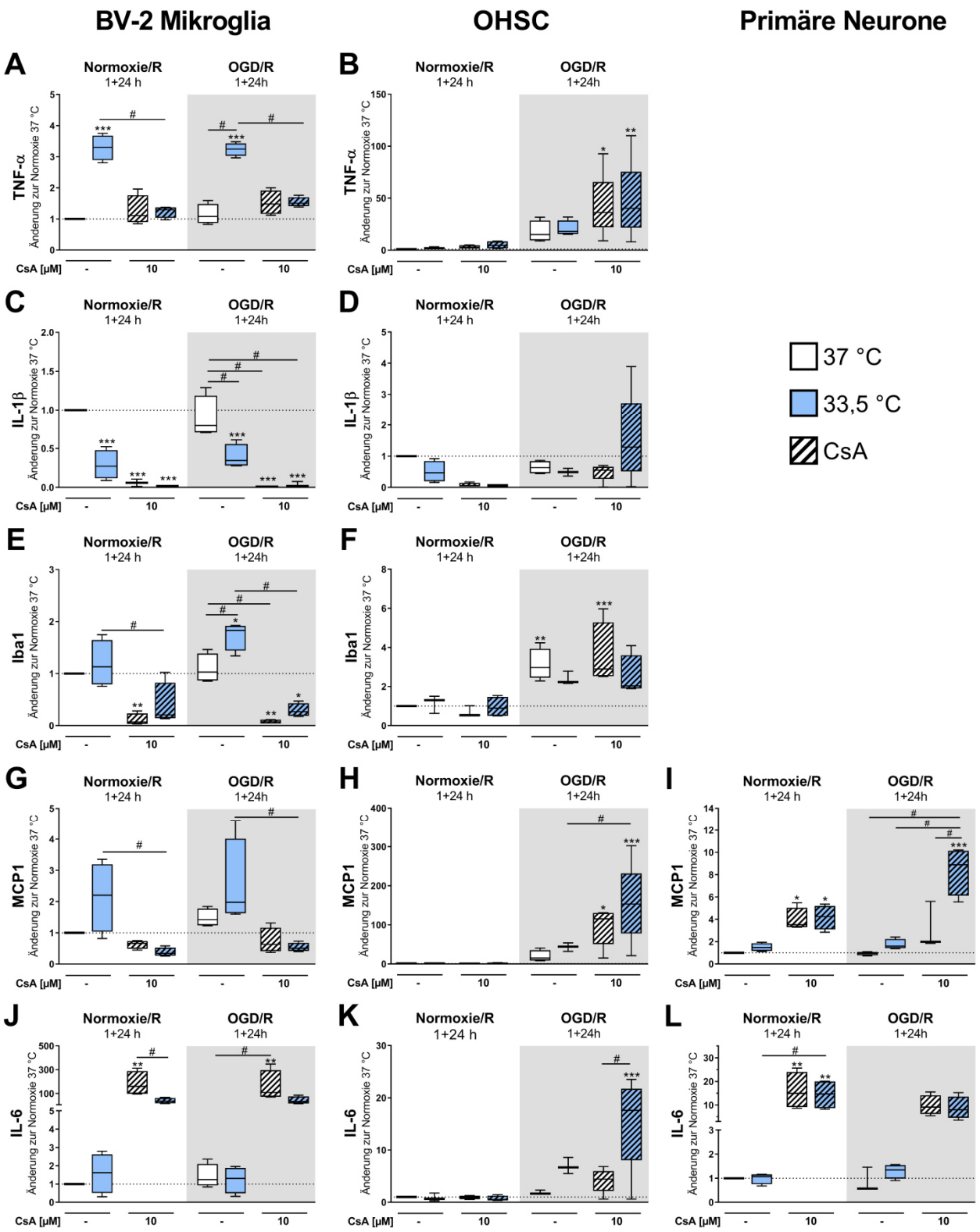

Abbildung 5. RT-qPCR zur Genexpressionsanalyse von (A/B) TNF-α, (C/D) IL-1β, (E/F) Iba1, (G/H/I) MCP1 und (J/K/L) IL-6 in BV-2 Mikroglia, organotypischen hippocampalen Schnittkulturen (OHSC) und primären Neuronen unter Kontrollbedingungen Normoxie/R (1h Normoxie (21 % O_2) + 24 h Reperfusion) bei 37 °C oder 33,5 °C und nach OGD/R (1h *Oxygen-Glucose-Deprivation* + 24 h Reperfusion) bei 37 °C oder 33,5 °C. Alle mit Cyclosporin A behandelten Gruppen wurden für eine Stunde vorbehandelt. Daten aus n= 3-5 unabhängigen Experimenten sind als Box-Whiskers-Plots dargestellt. Signifikante Unterschiede wurden mittels einseitiger ANOVA mit anschließendem Tukey *post-hoc*-Test berechnet und sind mit **p<0,01, ***p<0,001 (im Vergleich zu Normoxie, 37 °C) und mit #p<0,05 (im Gruppenvergleich) gekennzeichnet (modifiziert nach Wowro et al.[25]).

Die Transkription des Chemokins MCP1 wurde in den BV-2 Mikroglia nach Normoxie/R und OGD/R durch Kühlung leicht induziert, jedoch wurde diese durch 10 µM CsA signifikant im Vergleich zur gekühlten Gruppe reduziert (Abbildung 5 G). In den OHSC hingegen bewirkte OGD/R+CsA eine signifikante Erhöhung der MCP1-Expression gegenüber der Normoxie/R, die jedoch nicht bei der Normoxie/R+CsA beobachtet wurde. Auch die primären Neuronen exprimierten durch die CsA-Behandlung mehr MCP1 unter Normoxie/R und OGD/R (Abbildung 5 H/I). Die Expressionsmuster von IL-6 in den BV-2 Mikroglia und den primären Neuronen wiesen Ähnlichkeiten auf. Es kam zur signifikanten induzierte Transkription von IL-6 durch die Behandlung mit CsA bei 37 °C und zum Teil bei 33,5 °C nach Normoxie/R und OGD/R. In den OHSC war die Expression hingegen nur durch OGD/R+CsA bei 33,5 °C signifikant erhöht (Abbildung 5 J/K/L).

1.5.2 Publikation 2: Neuroprotektion durch die Expression von RBM3 in humanen SK-N-SH Neuronen wird durch Hypothermie, aber nicht Hypoxie reguliert [27]

Eine schwere Hypoxie (0,2 % O_2) für 24 h, jedoch nicht eine moderate Hypoxie (8 % O_2), gefolgt von einer 24-stündigen Reoxygenierung, führte in der humanen neuronalen Zelllinie SK-N-SH zu einem signifikanten Anstieg des LDH im Zellkulturüberstand. Der induzierte Schaden konnte durch Kühlung auf 33,5 °C für 48 h und 72 h nach der Hypoxie signifikant reduziert werden. Des Weiteren untersuchten wir den klinisch relevanten Biomarker für neuronale Schädigung NSE. Dieser war nach 24-stündiger schwerer Hypoxie, gefolgt von 72 h Normoxie, signifikant erhöht. Der neuronale Schaden konnte durch 72-stündige Kühlung der Zellen auf 33,5 °C signifikant reduziert werden. Die metabolische Aktivität der Zellen, gemessen am zellulären ATP-Gehalt, wurde durch die Inkubation bei schwerer Hypoxie signifikant reduziert. Durch Wiederherstellung des atmosphärischen O_2-Gehalts stieg die metabolische Zellaktivität wieder an und zeigte keinen Unterschied zu den ungeschädigten Vergleichsgruppen. Morphologisch konnte durch die Reduktion des Sauerstoffgehalts für 24 h auch noch nach 72 Stunden eine deutliche Schwellung der Zellen beobachtet werden. Besonders die schwere Hypoxie bewirkte eine deutliche Vergrößerung der Zelloberfläche im Vergleich zu den Kontrollgruppen bei 21 % O_2. Durch die folgende, 72-stündige Kühlung verminderte sich die Größe der Zellen wieder und nahm eine physiologische Zellmorphologie an. Die Expression des Kälteschockproteins RBM3 war in allen gekühlten Versuchsgruppen nach 48 bis 72 h sowohl auf mRNA- als auch auf Proteinebene im Vergleich zu den Kontrollgruppen bei 37 °C erhöht. Zudem war die Expression des RBM3 unabhängig von der vorangegangenen Hypoxie. Die Abbildungen der hier dargestellten Ergebnisse sind der Publikation von Rosenthal et al. zu entnehmen [27].

1.5.3 Publikation 3: Moderate therapeutische Hypothermie induziert protektive Mechanismen in Sauerstoff- Glukose-deprivierten und Reperfusion-geschädigten Kardiomyozyten [28]

Die Schädigung der Kardiomyozyten durch OGD/R bewirkte eine mitochondriale Dysfunktion, welche durch den Verlust des Membranpotentials mittels Mitotracker Red™ Färbung sowie durch die Ausschüttung von AIF und Cyto C ins Zytosol nachgewiesen werden konnte. Die frühe intraischämische Kühlung auf 33,5 °C konnte das Membranpotential erhalten. Zudem wurden durch die Kühlung zu den Reperfusionszeitpunkten nach 7, 8, 10 und 27 Stunden signifikant weniger Cyto C und nach 10, 12 und 27 Stunden signifikant weniger AIF ins Zytosol ausgeschüttet. Zur Untersuchung einer durch OGD/R-induzierten Apoptose wurde das Verhältnis von antiapoptotischen Bcl-2 zu proapoptotischen Bax mittels Western Blot-Analyse bestimmt. In den gekühlten Zellen wurde ein signifikanter Anstieg des Verhältnisses nach 8 und 27 Stunden gemessen. Das antiapoptotische Protein Hsp70 wurde durch die Kühlung nach sechsstündiger OGD signifikant erhöht exprimiert. Zudem wurde besonders in den frühen Zeitpunkten der OGD/R (nach 7, 8 und 9 Stunden) signifikant mehr LC3-II in der mitochondrialen Fraktion der gekühlten Gruppen im Vergleich zu ungekühlten Kontrollgruppen gemessen. Die Abbildungen der hier dargestellten Ergebnisse sind der Publikation von Krech et al. zu entnehmen [28].

1.6 Diskussion

Der Einsatz von Hypothermie hat sich zum Schutz vor Gewebeschädigung *in vitro* und *in vivo* bewährt und stellt besonders bei einer I/R-Schädigung im Gehirn und Herzen die effektivste Methode zum Schutz vor Zelluntergang dar. Neben dem Zelltod spielt die Neuroinflammation nach ischämischen Ereignissen in der Pathogenese zerebraler Schäden eine entscheidende Rolle [29].

Im Folgenden werden die Ergebnisse der drei ausgewählten Publikationen zusammengefasst diskutiert. Dabei stehen die Ergebnisse der Publikation 1 von Wowro et al. [25] im Vordergrund, in der die Wirkung von Hypothermie in Kombination mit dem immunsuppressiven CsA in OGD/Reperfusion-geschädigten zerebralen Zellen untersucht wurde. Die Gliederung erfolgt dabei nicht chronologisch nach den Publikationen, sondern beleuchtet zunächst die durch I/R-induzierte Schädigung und anschließend die Neuroinflammation.

1.6.1 Zelluläre Schädigung nach Ischämie/Reperfusion und Interventionsstrategien

In der neuronalen SK-N-SH Zelllinie wurde zunächst gezeigt, dass die 24-stündige Hypoxie bei 0,2 % O_2 zur Abnahme der metabolischen Aktivität führt. Die darauffolgende 24- bis 72-stündige Reoxygenierung hingegen bewirkte einen nekrotischen Zelluntergang, der durch Kühlung auf 33,5 °C signifikant vermindert werden konnte (Publikation 2: Rosenthal et al. [27]). RBM3 gehört zur Klasse der Kälteschockproteine und wird durch multiple Stressfaktoren zum Schutz der Zelle induziert [29]. In unserem Schädigungsmodell, in Analogie zur klinischen perinatalen Asphyxie, konnten wir ebenfalls eine durch Hypothermie induzierte Aktivierung der RBM3-Expression mit neuroprotektiver Wirkung feststellen. Ischämische Ereignisse führen zu einer komplexen Zellschädigung unter Alteration vieler Stoffwechselwege [30]. Ebenso wie die Ischämie wirkt Hypothermie global auf alle zellulären Mechanismen und bewirkt primär eine Reduktion des Zellstoffwechsels. Ungeachtet dessen kommt es zu einer verstärkte Expression der Kälteschockproteine, die somit einen möglichen Schutzmechanismus vor I/R-induziertem Zelltod darstellt [9]. Andere Studien konnten ebenfalls zeigen, dass RBM3 spezifisch durch Reduktion von apoptotischen Zelltod an der durch Hypothermie vermittelten Neuroprotektion beteiligt ist [16].

In der darauffolgenden Studie wurden Zellen des ZNS einer simulierten Ischämie ausgesetzt, wobei neben dem Sauerstoffentzug (0,2 % O_2) auch ein Glukose- und Serumentzug erfolgte (Publikation 1: Wowro et al. [25]). Eine einstündige OGD-Phase führte dabei weder in den Monokulturen aus primären Neuronen, in den BV-2 Mikroglia noch in den *ex vivo* OHSC zu einem signifikanten Anstieg des Zelltods. Die schnelle Wiederversorgung mit Sauerstoff und Nährstoffen und die damit einhergehende Dysbalance des Redox-Systems sowie die Beeinträchtigung zahlreicher intrazellulärer Prozesse kann zu einem Reperfusionsschaden führen [4]. Neurone gelten als stärker vulnerabel für Reperfusionsschäden als Gliazellen [31, 32]. Dies konnte in der vorliegenden Arbeit durch eine gesteigerte Zytotoxizität in den primären Neuronen und den OHSC bestätigt werden, wohingegen die Zytotoxizität in den BV-2 Mikroglia nicht anstieg [25]. Die Kühlung der primären Neurone auf 33,5 °C in der Reperfusionsphase

hatte keinen protektiven Effekt gegenüber der ungekühlten Gruppe, jedoch wurde tendenziell weniger Zelltod in den gekühlten OHSC detektiert. Dies kann auf eine protektive Wirkung der Hypothermie auf den komplexen Zellverband oder auch auf andere Zelltypen, wie Astrozyten, hinweisen. Andere Studien zeigten, dass eine frühe Kühlung bereits in der Ischämiephase das Überleben von Neuronen in OHSC deutlich verbessern kann [33], und auch in einem *in vivo* Schlaganfall-Modell resultierte eine intraischämische Kühlung in einen reduzierten Infarktareal [34]. Somit stellt die frühe Kühlung eine protektive Behandlungsstrategie dar.

In einem weiteren Zellkulturmodell aus Kardiomyozyten konnte gezeigt werden, dass OGD/R-Schädigung auch mit dem Verlust der mitochondrialen Membranintegrität einhergeht (Publikation 3: Krech et al. [28]). In Kardiomyozyten bilden Mitochondrien aufgrund des hohen Energiebedarfs bis zu 40 % des intrazellulären Volumens [35] und die Schädigung der Mitochondrien durch I/R hat einen großen Einfluss auf den Krankheitsverlauf. Unsere Studie zeigte, dass intraischämische Kühlung das Membranpotential erhält und die Ausschüttung der Apoptose-induzierenden Proteine Cyto C und AIF aus den Mitochondrien signifikant reduziert (Krech et al. [28]). Auch Huang et al. konnten zeigen, dass Hypothermie die mitochondriale Dysfunktion nach I/R vermindern kann [36]. Zudem wurden die antiapoptotischen Proteine Hsp70, das Caspase-abhängige und -unabhängige Apoptose inhibieren kann [37] und Bcl-2, das die äußere mitochondriale Membran stabilisiert [38], unter Kühlung verstärkt exprimiert. Damit stellt der Erhalt der mitochondrialen Integrität einen weiteren, durch Kühlung vermittelten Schutzmechanismus dar. Das bestätigt die Hypothese, dass eine frühe Kühlung das Zellüberleben nach I/R-Schädigung fördert.

Der Wirkstoff CsA wird derzeit als potentiell neuroprotektive Substanz erforscht und diskutiert. Ein Grund dafür ist die immunsuppressive Wirkung durch die Inhibition der Kalzium/Calmodulin abhängigen Proteinphosphatase Calcineurin. Sie wird in hoher Konzentration in Zellen des ZNS exprimiert und spielt als Aktivator multipler, proinflammatorischer Transkriptionsfaktoren wie dem a*ctivator protein 1* (AP-1), NFAT und dem *nuclear factor kappa-light-chain-enhancer of activated B-cells* (NF-κB) ein Rolle in der Neuroinflammation [39]. Des Weiteren bindet CsA an Cyclophilin D und vermindert dadurch die Bildung einer mPTP. Diese Pore in der inneren Mitochondrienmembran ist maßgebend im I/R-vermittelten Zelltod, da deren Öffnung zum Verlust des Membranpotentials und dadurch zur Abnahme von ATP, zur Schwellung der Mitochondrien und zur Produktion von reaktiver Sauerstoffspezies führt. Die Ausbildung der mPTP nach der I/R-induzierten Schädigung kann neben der Apoptose auch Autophagie und Nekrose induzieren [23].

Wie im Kardiomyozyten I/R Modell gezeigt, kann Hypothermie eine mitochondriale Dysfunktion vermindern [28]. Die Kombination von Hypothermie mit CsA wurde ebenfalls als protektiv auf neuronale Mitochondrien beschrieben [40]. Immunregulatorische Eigenschaften der Kombinationstherapie wurden bisher jedoch noch nicht beschrieben. Wir konnten zunächst zeigen, dass es in den primären Neuronen und OHSC durch die Behandlung mit 10 µM CsA zu einem Anstieg der Zytotoxizität kommt. Jedoch

wurde der beobachtete Reperfusionsschaden in beiden Zellmodellen weder durch die Behandlung mit CsA noch durch Kühlung reduziert oder verstärkt. In den BV-2 Mikroglia hingegen war CsA in der OGD-Phase zunächst nicht zytotoxisch, aber nach der 24-stündigen Reperfusionsphase war eine konzentrationsabhängige CsA-Schädigung zu detektieren, welche durch gleichzeitige Kühlung tendenziell gemindert wurde [25]. Auch Kaminska et al. zeigten, dass die Behandlung mit 8 µM CsA in einer Neuronen-Astrozyten-Mischkultur einen zytotoxischen Effekt auf Neurone, nicht aber auf die Astrozyten hat [41]. Somit erwiesen sich Gliazellen als robuster gegenüber der Behandlung mit CsA als Neurone.

1.6.2 Inflammation nach Ischämie/Reperfusion und Interventionsstrategien

Die Neuroinflammation nach einem I/R-Ereignis wird unter anderem durch DAMPs, die von nekrotischen Zellen ausgeschüttet werden, initiiert und verstärkt. Zu dieser Gruppe der DAMPs zählen unter anderem die beiden in der Literatur gut beschriebenen Proteine Hsp70 und HMGB1 sowie das erst kürzlich als DAMP identifizierte Kälteschockprotein CIRBP. Intrazellulär weisen Hsp70 und CIRBP antiapoptotische Eigenschaften auf, können jedoch extrazellular eine Inflammationsreaktion hervorrufen [42, 43]. Durch die Bindung am *receptor for advanced glycation end products* sowie am TLR2 und TLR4 an benachbarten Zellen werden Inflammationskaskaden über den Transkriptionsfaktor NF-κB initiiert, die zur Transkription von TNF-α, IL-1β und IL-6 führen [44, 45]. In Korrelation mit der Zytotoxizität konnte in der hier vorliegenden Arbeit extrazellulär ein signifikanter Anstieg der Proteine Hsp70 und HMGB1 sowie ein tendenzieller Anstieg von CIRBP im Überstand der primären Neurone nach OGD+CsA, jedoch nicht bei den BV-2 Mikroglia, gegenüber der Normoxie-Gruppe und der OGD-Gruppe nachgewiesen werden. Zou und Crews konnten zeigen, dass DAMPs von nekrotischen Neuronen in hippocampalen Schnitten, sowie die Behandlung mit HMGB1 über den TLR4 die Expression von TNF-α und IL-1β in Mikroglia bewirken [46]. Da in der vorliegenden Studie auch ein Zelluntergang in den OHSC nach OGD+CsA nachweisbar war, kann vermutet werden, dass auch DAMPs ausgeschüttet werden und benachbarte Zellen aktivieren. Aufgrund hoher Serumkonzentrationen in den Reperfusionsmedien war ein Nachweis der DAMPs über Western Blot-Analysen in den Zellkulturmodellen nicht möglich.

In dieser Arbeit wurde die immunregulatorische Wirkung der Kühlung und der CsA-Behandlung in den Monokulturen von Mikroglia und Neuronen untersucht. Gekühlte BV-2 Mikroglia exprimierten verstärkt TNF-α und MCP1, wohingegen IL-1β inhibiert wurde. Während das Zytokin IL-1β mit neurotoxischen Effekten assoziiert ist, kann TNF-α auch neuroprotektive Wirkung aufweisen [47]. In einem *in vivo* Modell von zerebraler Ischämie wurde gezeigt, dass von Mikroglia sezerniertes TNF-α das Überleben von Neuronen fördert [48]. Zusätzlich kann eine durch Kühlung induzierte TNF-α Sekretion das Neuritenwachstum in *ex vivo* OHSC fördern [49]. Jedoch zeigen hohe TNF-α-Spiegel im ischämisch geschädigten Gehirn auch neurotoxische Wirkung [50]. Das Chemokin MCP1 ist primär für die Rekrutierung peripherer Leukozyten zum geschädigten Gewebe verantwortlich. Während eine Invasion der Leukozyten zunächst als protektiv galt, kann sie auch zur Verschlechterung des Hirnschadens

beitragen. So erwies sich die Inhibition der MCP1-Expression in einem *in vivo* Schlaganfall-Modell durch Abnahme von ischämischen Hirnschäden als protektiv [51].

Bei gleichzeitiger Behandlung mit CsA wurde die durch Kühlung induzierte Expression des MCP1 sowie des TNF-α in den BV-2 Mikroglia reduziert. Zudem vermittelte CsA einen inhibitorischen Effekt auf die Expression von Iba1, einem Marker für aktivierte Mikroglia, und supprimierte signifikant die IL-1β-Expression. Dieses deckt sich mit der Arbeit von Zawadzka et al., die in einem LPS-aktivierten *in vitro* Mikroglia-Modell die inhibitorische Wirkung von CsA auf die MAPK- und NF-κB-Signalkaskaden zeigten [52]. Dies ist nicht überraschend, da Calcineurin indirekt durch Abbau von IκBα an der Aktivierung von NF-κB beteiligt ist. Zudem wird die Expressionen der Zytokine über Komplexe von den Transkriptionsfaktoren NFAT, NF-κB und AP-1 reguliert [53-55]. Spezifische NFAT-Inhibitoren sind beispielsweise ebenfalls in der Lage die Expression von MCP1 und TNF-α in Mikroglia zu reduzieren [56].

Neben der suppressiven Wirkung von CsA auf die BV-2 Mikroglia wurde zusätzlich ein signifikanter Anstieg der IL-6-Genexpression induziert. Das Zytokin wird von immunkompetenten, aber auch nicht immunkompetenten Zellen wie den Neuronen sekretiert und kann als neurotrophischer Faktor den Zelluntergang von Neuronen inhibieren [57]. Jedoch sind hohe IL-6-Level auch an der Pathogenese vieler inflammatorischer Erkrankungen beteiligt [58]. In den primären Neuronen kam es zu einem signifikanten Anstieg der Expression von IL-6 durch die Behandlung mit 10 µM CsA, jedoch nicht durch OGD/R allein. Ähnlich war die Expression von MCP1 in den primären Neuronen durch 10 µM CsA, jedoch nicht durch OGD/R alleine induziert. Demnach scheint die alleinige Applikation von CsA proinflammaorisch auf die primären Neurone zu wirken. So wurde auch gezeigt, dass eine neuronale Expression von MCP1 eine Aktivierung von Mikroglia bewirken kann [59].

Der durch CsA vermitttelte inhibitorische Effekt auf die BV-2 Mikroglia und die cytotoxische und inflammatorisch-aktivierende Wirkung in den primären Neuronen wurden zusätzlich in einem komplexen OHSC-Modell untersucht. Der Vorteil liegt dabei in den aufrechterhaltenen Zell-Zell-Verbindungen und der Untersuchung von komplexen Interaktionen verschiedener Zelltypen. Die OGD/R-Schädigung führte in dem OHSC-Modell lediglich zur signifikanten Induktion von Iba1. Bei Behandlung mit 10 µM CsA während der OGD/R wurde zudem die Expression von TNF-α, MCP1 und Iba1 signifikant induziert. Die zusätzliche Kombination mit Hypothermie induzierte zudem die Expression von IL-6. Dagegen hatten CsA und Hypothermie keinen Einfluss auf die ungeschädigte Normoxie/R-Kontrollgruppe. Demnach induzieren CsA und Hypothermie nur in I/R-geschädigten OHSC eine Inflammation.

Neben den Neuronen und Mikroglia machen die Astrozyten die größte Zellpopulation im Gehirn aus. Sie übernehmen neben der Stütz- und Versorgungsfunktion auf vielseitige Art eine protektive Rolle im Gehirn und sind ausgiebig an der Immunantwort beteiligt [60]. Die gemessene Inflammationsreaktion in den OHSC kann somit auch zum Teil durch die Astrozyten vermittelt worden sein. Eine erhöhte MCP1-Expression wurde zum Beispiel in hippocampalen Astrozyten in einem *in vivo* Modell von transienter

globaler Ischämie beobachtet [61] und dies kann die adaptive Immunreaktion im entzündeten Gehirn verstärken [62]. Andere Studien, die eine neuroprotektive Wirkung von CsA untersuchten, zeigten unter anderem, dass die Behandlung von OGD-geschädigten primären Astrozyten mit CsA die Expression von TNF-α und IL-1β vermindern kann [63]. Zudem zeigte eine *in vivo* Studie, dass die Behandlung mit CsA-haltigen Nanoliposomen nach einem transienten Verschluss der mittleren Zerebralarterie das Infarktareal vermindert und die TNF-α-Sekretion im Vergleich zu nicht CsA-behandelten Tieren reduziert [64]. Diese Untersuchungen aus den Astrozyten und den *in vivo* Modellen stehen im Kontrast zu unseren Beobachtungen nach OGD/R+CsA. Es ist nicht auszuschließen, dass der putativ protektive Effekt von CsA in unserem Zellkulturmodellen durch komplexe, gegenseitige inflammatorische Aktivierung überlagert und dadurch verhindert wird.

1.6.3 Zusammenfassung und Ausblick

In der ersten Studie (Wowro et al. [25]) konnte durch die Kombination von Hypothermie und CsA-Behandlung eine Immunmodulation gezeigt werden. CsA konnte die proinflammatorische Aktivität von BV-2 Mikroglia nach OGD/R vermindern, hatte in höheren Konzentrationen aber einen toxischen Effekt auf die primären Neurone. Während die Kombination von CsA und Kühlung die Expression des proinflammatorischen IL-1β in den BV-2 reduzierte, war eine Induktion von TNF-α, MCP1, IL-6 und Iba1 in OGD/R-geschädigten OHSC messbar. In den primären Neuronen kam es neben dem nekrotischen Zelluntergang zur CsA-induzieren Expression von IL-6 und MCP1. Die von den nekrotischen Zellen verstärkt ausgeschütteten DAMPs und proinflammatorischen Zytokine können einen komplexen sekundären Schädigungsmechanismus mit Inflammationsreaktion in den OHSC induzieren. So kommt es durch die Kombinationstherapie wider Erwarten nicht zu einem additiven Schutzmechanismus in dem komplexen *ex vivo* Modell, sondern zu einer starken Inflammationsreaktion.

Mechanistisch konnte in der zweiten Studie an einer neuronalen Zelllinie gezeigt werden, dass der protektive Effekt der Kühlung mit der induzierten Expression vom Kälteschockprotein RBM3 korreliert (Rosenthal et al. [27]). Zudem konnte in der dritten Studie gezeigt werden, dass die intraischämische Kühlung die Integrität der mitochondrialen Membran in Kardiomyozyten erhalten kann, wodurch die intrinsische Apoptose reduziert und Autophagiemechanismen induziert werden (Krech et al. [28]).

Damit konnten wir in Monozellkulturmodellen zeigen, dass Hypothermie nach einem ischämischen Schädigungsereignis protektive Mechanismen aufweist. Diese führen in den primären Neuronen interessanterweise nicht zum Schutz vor OGD/R-induzierter Schädigung. Um ein genaueres Bild von den Vorgängen in den OHSC zu bekommen, muss jedoch in weiteren Studien zusätzlich der Einfluss der Astrozyten untersucht werden. Zudem ist der Nachweis sezernierter Zytokine und die Ausschüttung von DAMPs nach der OGD/R-Schädigung für weitere Schlussfolgerungen notwendig. Aufgrund der wichtigen Rolle der Immunreaktion nach ischämischen Ereignissen ist die Untersuchung von Ko-Kulturen, Gewebekulturen und *in vivo* Modellen für ein besseres Verständnis der Mechanismen von Kühlung unumgänglich.

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2 Eidesstattliche Versicherung

„Ich, Sylvia J. Wowro, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Hypothermie-induzierte zelluläre und molekulare Mechanismen in ischämisch geschädigten Zellen“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

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Datum Unterschrift

3 Anteilserklärung an den erfolgten Publikationen

Sylvia J. Wowro hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1 (Wowro et al. 2019):

Wowro SJ, Tong G, Krech J, Rolfs N, Berger F, Schmitt KRL. „Combined cyclosporin A and hypothermia treatment inhibits activation of BV-2 microglia but induces an inflammatory response in an ischemia/reperfusion hippocampal slice culture model." Frontiers in Cellular Neuroscience, 06/ 2019, doi: 10.3389/fncel.2019.00273. Impact Factor: 4.3.

Beitrag im Einzelnen:

- Erstellung des Versuchsdesign
- Präparation der primären Neurone und Präparation und Etablierung der Kultivierung von organotypischen hippocampalen Schnittkulturen
- Durchführung der Experimente (qPCR, Färbung, Western Blot, LDH)
- Datenauswertung und Darstellung der Ergebnisse
- Datenanalyse
- Literaturrecherche
- Fertigung des Manuskriptentwurfs
- Korrespondierender Autor

Publikation 2 (Rosenthal et al. 2017):

Rosenthal LM, Tong G, Walker C**, Wowro SJ**, Krech J, Pfitzer C, Justus G, Berger F, Schmitt KRL. „Neuroprotection via RNA-binding protein RBM3 expression is regulated by hypothermia but not by hypoxia in human SK-N-SH neurons." Hypoxia, 05/ 2017, 5:33-43. Impact Factor: N/A.

Beitrag im Einzelnen (geschätzter Anteil an der finalen Publikation: 20%):

- Unterstützung bei der Vorbereitung und Durchführung der Experimente der Medizinstudentin Lisa Rosenthal
- Betreuung der Zellkulturarbeiten
- Datenanalyse und Unterstützung in der Darstellung und Präsentation der Ergebnisse
- Diskussion der Ergebnisse
- Korrekturlesen des Manuskripts

Publikation 3 (Krech et al. 2017):

Krech J, Tong G, **Wowro S,** Walker C, Rosenthal LM, Berger F, Schmitt KRL. „Moderate therapeutic hypothermia induces multimodal protective effects in oxygen-glucose deprivation/reperfusion injured cardiomyocytes." Mitochondrion, 07/ 2017, 35:1-10. Impact Factor: 3.2.

Beitrag im Einzelnen (geschätzter Anteil an der finalen Publikation: 25%):

- Einarbeitung und Betreuung der Zellkulturexperimente der Medizinstudentin Jana Krech
- Anleitung, Planung und Mitdurchführung der Versuche (Proteingewinnung Mitochondrien/Zytosol, Immunfluoreszenz Färbung)
- Datenanalyse und Diskussion der in die Publikation integrierten Ergebnisse
- Hilfe bei der Erstellung der Abbildungen und Korrektur des Manuskripts

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Unterschrift des Doktoranden/der Doktorandin

4 Druckexemplare der ausgewählten Publikationen

4.1 Publikation 1 (Wowro et al. 2019)

Wowro SJ, Tong G, Krech J, Rolfs N, Berger F, Schmitt KRL.

„Combined cyclosporin A and hypothermia treatment inhibits activation of BV-2 microglia but induces an inflammatory response in an ischemia/reperfusion hippocampal slice culture model."

Frontiers in Cellular Neuroscience, 06/ 2019, doi: 10.3389/fncel.2019.00273.

Impact Factor: 4.3

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 25 June 2019
doi: 10.3389/fncel.2019.00273

Combined Cyclosporin A and Hypothermia Treatment Inhibits Activation of BV-2 Microglia but Induces an Inflammatory Response in an Ischemia/Reperfusion Hippocampal Slice Culture Model

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OPEN ACCESS

Edited by:
Xin Qi,
Case Western Reserve University, United States

Reviewed by:
Peter Racay,
Comenius University, Slovakia
Sharon DeMorrow,
The University of Texas at Austin, United States

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Specialty section:
This article was submitted to Cellular Neuropathology, a section of the journal Frontiers in Cellular Neuroscience

Received: *27 March 2019*
Accepted: *05 June 2019*
Published: *25 June 2019*

Citation:
Wowro SJ, Tong G, Krech J, Rolfs N, Berger F and Schmitt KRL (2019) Combined Cyclosporin A and Hypothermia Treatment Inhibits Activation of BV-2 Microglia but Induces an Inflammatory Response in an Ischemia/Reperfusion Hippocampal Slice Culture Model. Front. Cell. Neurosci. 13:273. doi: 10.3389/fncel.2019.00273

Introduction: Hypothermia attenuates cerebral ischemia-induced neuronal cell death associated with neuroinflammation. The calcineurin inhibitor cyclosporin A (CsA) has been shown to be neuroprotective by minimizing activation of inflammatory pathways. Therefore, we investigated whether the combination of hypothermia and treatment with CsA has neuroprotective effects in an oxygen-glucose deprivation/reperfusion (OGD/R) injury model in neuronal and BV-2 microglia monocultures, as well as in an organotypic hippocampal slice culture (OHSC).

Methods: Murine primary neurons, BV-2 microglia, and OHSC were pretreated with CsA and exposed to 1 h OGD (0.2% O_2) followed by reperfusion at normothermia (37°C) or hypothermia (33.5°C). Cytotoxicity was measured by lactate dehydrogenase and glutamate releases. Damage-associated molecular patterns (DAMPs) high mobility group box 1 (HMGB1), heat shock protein 70 (Hsp70), and cold-inducible RNA-binding protein (CIRBP) were detected in cultured supernatant by western blot analysis. Interleukin-6 (IL-6), Interleukin-1α and -1β (IL-1α/IL1-β), tumor necrosis factor-α (TNF-α), monocyte chemotactic protein 1 (MCP1), inducible nitric oxide synthase (iNOS), glia activation factors ionized calcium-binding adapter molecule 1 (Iba1), and transforming growth factor β1 (TGF-β1) gene expressions were analyzed by RT-qPCR.

Results: Exposure to OGD plus 10 μM CsA was sufficient to induce necrotic cell death and subsequent release of DAMPs in neurons but not BV-2 microglia. Moreover, OGD/R-induced secondary injury was also observed only in the neurons, which was not attenuated by cooling and no increased toxicity by CsA was observed. BV-2 microglia were not sensitive to OGD/R-induced injury but were susceptible to CsA-induced toxicity in a dose dependent manner, which was minimized by hypothermia. CsA attenuated IL-1β and Iba1 expressions in BV-2 microglia exposed

to OGD/R. Hypothermia reduced IL-1β and iNOS expressions but induced TNF-α and Iba1 expressions in the microglia. However, these observations did not translate to the *ex vivo* OHCS model, as general high expressions of most cytokines investigated were observed.

Conclusion: Treatment with CsA has neurotoxic effects on primary neurons exposed to OGD but could inhibit BV-2 microglia activation. However, CsA and hypothermia treatment after ischemia/reperfusion injury results in cytotoxic neuroinflammation in the complex *ex vivo* OHSC.

Keywords: cyclosporin A, hypothermia, oxygen-glucose deprivation/reperfusion, organotypic hippocampal slice culture, BV-2 microglia, primary neuron, inflammation, DAMPs

INTRODUCTION

Therapeutic hypothermia (TH), also referred to as targeted temperature management (TTM), is a clinically established strategy for neuroprotection against ischemia/reperfusion injury. Several clinical studies in newborns and infants suffering from perinatal asphyxia and neonatal encephalopathy have demonstrated the efficacy of cooling to 33–34°C, resulting in lower mortality rates and improved neurological outcomes (Shankaran et al., 2012; Azzopardi et al., 2014). Additionally, cooling to a targeted temperature of 32–36°C has been established as a standard of care in patients after out-of-hospital cardiac arrest to reduce risk of death and improve neurological outcome (Hypothermia after Cardiac Arrest Study, 2002; Callaway et al., 2015; Nolan et al., 2015). Accumulating data also promotes hypothermia as a promising neuroprotective strategy in rodent models of traumatic brain injury (Liu et al., 2016b; Zhao et al., 2017) and stroke (Lee et al., 2016; Liu et al., 2018), but have yet to be translated to human trials. The recent Prophylactic Hypothermia Trial to Lessen Traumatic Brain Injury–Randomized Clinical Trial (POLAR-RCT) trial reported no benefits in neurological outcome at 6 months after applying early moderate hypothermia after severe traumatic brain injury (Cooper et al., 2018) and an acute ischemic stroke trial (Intravascular Cooling in the Treatment of Stroke) reported increased incidents of pneumonia and mortality in the hypothermia vs. normothermia treated group (Lyden et al., 2016).

Experimental investigations on the cytoprotective effects of hypothermia implicate a complex multi-modal response to protect from various ischemia/reperfusion injury mechanisms, including calcium influx, oxidative stress, mitochondrial dysfunction, apoptosis, excitotoxicity (neuronal death), and inflammation (Krech et al., 2017; Kurisu and Yenari, 2018), but the complete mechanism underlying hypothermia-induced neuroprotection remains to be elucidated. Additionally, these studies also did not investigate the effects of hypothermia on the injury-induced sterile inflammatory response.

In contrast to inflammation induced by pathogens, a sterile inflammatory response is induced by an acute condition, such as ischemia/reperfusion injury, in the absence of pathogens. Necrotic cells and other irritant particles are the stimuli for sterile inflammation by activating NLRP3 inflammasomes and inducing the release of interleukin-1 (IL-1), however, the complete mechanism is still not fully understood (Rock et al., 2010; Kono et al., 2014). Increasing evidence points to a sterile inflammatory response within the first few days after an ischemic insult that can exacerbate neuronal cell death (Ceulemans et al., 2010; Jin et al., 2013). Necrotic cell death due to an acute hypoxic-ischemic incident results in the release of DAMPs into the extracellular matrices, where they bind to pattern recognition receptors (e.g., TLRs) on local immunocompetent glial cells and initiate a neuroinflammatory response (Gulke et al., 2018).

CsA is an immunosuppressor used primarily in transplantation medicine to suppress the activation of T-lymphocytes (Borel et al., 1976). CsA binds to intracellular cyclophilin A and inhibits calcineurin, which regulates the immune response by modulating transcription factors activity (Clipstone and Crabtree, 1992). CsA has also been intensively discussed as a potential neuroprotectant as it inhibits the formation of the mitochondrial permeability transition pore by binding to cyclophilin D, thereby preventing mitochondrial dysfunction and apoptosis (Crompton, 1999; Osman et al., 2011; Fakharnia et al., 2017). Therefore, we investigated the anti-neuroinflammatory and neuroprotective effect of clinically established hypothermia in combination with CsA treatment in a murine primary neurons and BV-2 microglia monoculture model of simulated ischemia/reperfusion-induced injury. Additionally, we developed a complex murine OHSC simulated ischemia/reperfusion model where the tissue structures and cell interactions are preserved, and which is well suited for studying cell death and neuroprotective agents *ex vivo* (Li et al., 2016).

Abbreviations: AP1, activator protein 1; CIRBP, cold-inducible RNA-binding protein; CNS, central nervous system; CsA, cyclosporin A; DAMP, damage-associated molecular pattern; DIV, days *in vitro*; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMGB1, high mobility group box 1; Hsp70, heat shock protein 70; iNOS, inducible nitric oxide synthase; Iba1, ionized calcium-binding adapter molecule 1; IL-6, interleukin-6; IL-1α, interleukin-1α; IL-1β, interleukin-1β; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MCP1, monocyte chemotactic protein 1; NFAT, nuclear factor of activated T-cells; OGD, oxygen-glucose-deprivation; OGD/R, OGD and reperfusion; OHSC, organotypic hippocampal slice culture; PI, propidium iodide; PLL, poly-L-lysin; RAGE, receptor for advanced glycation endproducts; RT-qPCR, reverse transcription quantitative polymerase chain reaction; TGF-β1, transforming growth factor-β1; TH, therapeutic hypothermia; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α; TTM, targeted temperature management.

MATERIALS AND METHODS

Cell Culture

BV-2 microglial cells are immortalized murine microglial cells (Blasi et al., 1990) with a phenotype functionally identical to native primary microglia (Henn et al., 2009), and were a kind gift from Prof. Ullrich (Zürich, Switzerland). BV-2 cells were cultured in high glucose Dulbecco's Modified Eagle's Medium supplemented with 1 mM pyruvate (Biochrom), 10% heat inactivated fetal bovine serum (Biochrom), and 100 U/ml penicillin/100 µg/ml streptomycin (Merck Millipore) in a 5% CO_2 humidified atmosphere at 37°C. Cells were seeded 24 h prior to experimental start at a density of 500,000 cells in a 60 mm (21 cm^2) dish (Sarstedt) pre-coated with 10 µg/ml PLL (Sigma-Aldrich).

Animals and Preparation of Primary Cultures

All animal experiments were approved and performed in accordance with the guidelines of the Charité – Universitätsmedizin Berlin, Germany, and animals were housed in a conventional animal facility (FEM, Charité – Universitätsmedizin Berlin, Germany).

Preparation of Primary Neurons

Primary neurons were prepared from embryonic day 15 (E15) C57BL/6N mice with slight modifications to the protocol previously described (Schmitt et al., 2006). Briefly, after removal of the meninges, cerebral cortices and hippocampi were digested in Hanks Balanced Salt Solution (Thermo Fisher Scientific), containing 0.2% Trypsin (Biochrom) and 100 µg/ml DNase I (Roche Diagnostics), and dissociated with a glass pipette. Cells were initially plated on 200 µg/ml PLL (Sigma-Aldrich; in 0.1 M borate buffer) pre-coated 35 mm dishes (9.2 cm^2, TPP) in Minimum Essential Medium (MEM, Gibco) supplemented with 10% heat-inactivated horse serum (Biochrom), 6 g/L glucose (B. Braun), and 1 mg/ml Primocin (InvivoGen). The plating medium was changed to serum free Neurobasal® Medium (Gibco) supplemented with 1x B-27™ (Gibco), 0.5 mM L-glutamine, and 1 mg/ml Primocin. After 5 days *in vitro* (DIV5) medium was changed to Neurobasal® Medium supplemented with 1x B-27™ minus antioxidants (Gibco). Primary neurons were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Preparation of Organotypic Hippocampal Slice Cultures

Hippocampal slice cultures were prepared from C57BL/6N mice at postnatal day 3–5, with slight modifications to the protocol previously described (Schmitt et al., 2007). Briefly, mice were sacrificed by decapitation and the hippocampi were quickly removed and placed in ice cold MEM supplemented with 2.2 g/L sodium bicarbonate, 2 mM L-glutamine, and 8 mM Tris base (all Merck Millipore; pH 7.2). The hippocampi were transversely cut into 350 µm thick slices using a tissue chopper (McIlwain) and 6–8 slices were randomly distributed onto 30 mm membrane inserts with 0.4 µM pore size (Merck Millipore). The inserts were placed in a six-well culture dish with 1.3 ml culture medium, containing MEM, 20% heat-inactivated horse serum, 30 mM hepes (Biochrom), 2 mM L-glutamine, 2.2 g/L sodium bicarbonate, 1 µg/ml insulin (Insuman Rapid), 2.3 g/L glucose, 0.1 mg/ml Primocin, and 88 µg/ml vitamin C (Rotexmedica) at pH 7.2. Slices were cultured at 37°C in a humidified atmosphere with 5% CO_2 for 14 days and medium was changed 1 day after preparation and every second to third day thereafter.

Oxygen-Glucose Deprivation and Reperfusion (OGD/R)

Ischemia was simulated *in vitro* by incubation for 1 h in pre-equilibrated glucose and serum free medium at 0.2% O_2, 5% CO_2, and 94.8% N_2 in a CO_2 incubator (Binder). Control cells were maintained in glucose containing medium at Normoxia for 1 h (21% O_2). OGD exposure in primary neurons (DIV7) and OHSC (DIV14) was conducted in glucose-free Neurobasal®-A Medium and in BV-2 microglia in glucose-free DMEM. After OGD, reperfusion was simulated by restoration of glucose, serum and 21% O_2 for 24 h in all groups in appropriate complete medium.

Time-Temperature Protocol

An experimental time-temperature protocol is illustrated in **Figure 1**. All cultures were exposed to OGD (0.2% O_2) at normothermic temperature (37°C) for 1 h, followed by either normothermic (37°C) or moderate hypothermic (33.5°C) simulated reperfusion (21% O_2) for 24 h. Control groups were maintained under normoxic and normothermic conditions (21% O_2 and 37°C) for the duration of the experiment.

Cyclosporin A Treatment

Cyclosporin A was purchased from Cayman (#12088). A 20 mM stock solution dissolved in ethanol (EtOH) was aliquoted and stored at −20°C. Working CsA dilutions (1 or 10 µM) in appropriate culture medium were prepared freshly and used to pre-treat cultures at 37°C and 21% O_2 for 1 h prior to exposure to OGD and maintained throughout the duration of the experiment. Control cells were incubated with equal amount EtOH solvent.

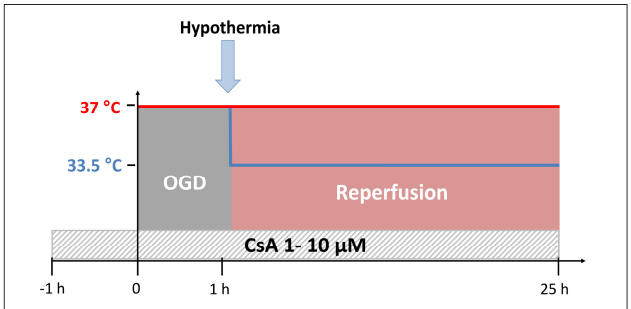

FIGURE 1 | Experimental time-temperature protocol. Cyclosporin A (CsA) was applied 1 h before the simulated ischemia phase by oxygen-glucose-deprivation (OGD at 0.2% O_2 in glucose-depleted medium) and maintained after the reperfusion phase for the duration of experiment. Exposure to OGD for 1 h was performed at 37°C, followed by reperfusion (OGD/R) for 24 h at 37°C (normothermia) or 33.5°C (hypothermia).

Assessment of Lactate Dehydrogenase (LDH) Release

Cell death was assessed by LDH released into the cultured supernatant using a colorimetric Cytotoxicity Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. Briefly, cultured supernatants were separated from cells by centrifugation and mixed with reagents (1:1) in a 96-well plate. Extinction was measured at 490 nm minus 630 nm using a microtiter plate reader (Thermo Fisher Multiskan Ascent). LDH release is expressed as a percentage of total LDH content, as determined from lysed normoxic control cells using a freeze/thaw method at −80°C.

Assessment of Glutamate Release

Glutamate release into the cultured supernatant was measured using the Glutamat-Glo™ Assay (Promega) according to the manufacturer's instruction. Briefly, cell free supernatant was incubated with glutamate detection reagent (1:1) in a 96-well plate and luminescence signal was measured using a microplate reader (Tecan Infinite® M200 Pro). Glutamate concentration was extrapolated from a concentration curve and expressed in μM.

Assessment of Propidium Iodide (PI) Staining in the OHSCs

Cell death in the OHSCs was assessed by PI staining. OHSCs were incubated with 4 μg/ml solution of PI (Sigma-Aldrich, Germany) for the last 30 min of the reperfusion phase. After staining, samples were washed twice with PBS and documented using a Keyence BZ-9000 inverted microscope.

Extracellular Proteins Isolation and Western Blot Analysis

Supernatants were collected after 1 h of OGD or Normoxia and extracellular proteins were isolated by trichloroacetic acid precipitation. Briefly, supernatants were incubated with 20% trichloroacetic acid (VWR) for 30 min on ice, and then centrifuged at 16,000 × *g* for 20 min. The precipitated proteins were washed with ice cold aceton and dissolved in RIPA buffer. Samples were incubated with Pierce Lane Marker Reducing Sample Buffer (Thermo Scientific) at 95°C for 5 min and subjected to a 15% SDS polyacryl gel electrophoresis. Proteins were transferred onto a polyvinylidene fluoride membrane (PALL Life Sciences) overnight at 30 V using a tank blot procedure (Bio-Rad Laboratories). The membrane was blocked for 1 h at room temperature using 5% bovine albumin fraction V (Carl Roth) for Hsp70 and HMGB1 or 5% dry milk (Applied Biosystems) for CIRBP in TBS + 0.1% Tween 20. Primary antibodies against Hsp70 (1:1000, Cell Signaling Technology, Cat#4872) HMGB1 (1:1000, Chondrex, Cat#7028), and CIRBP (1:1000, Abclonal, Cat#A6080) were diluted in blocking solution and blots were incubated overnight at 4°C. HRP-conjugated secondary antibodies (anti-rabbit IgG, 1:20,000 Dianova) were incubated on the blots for 1 h at room temperature. We used Dura Super Signal West (Thermo Fisher Scientific) to visualize protein expression and ChemiDoc™ Imaging Systems and Image Lab™ Software (Bio-Rad) for densitometry analysis.

RNA Isolation and Semi-Quantitative Real-Time qPCR

Total RNA from BV-2 microglia and primary neurons was isolated by acidic phenol/chloroform extraction using RNA Pure™ (Peqlab) and RNA from OHSC was isolated by using the GenUP™ Total RNA Kit (Biotechrabbit) followed by genomic DNA digestion using Turbo DNA-free™ Kit (Ambion) according to manufacturers' instructions, respectively. RNA concentration and purity was determined by spectrophotometric measurements at 260 nm and 280 nm using a Nanodrop 2000 (Nanodrop) and agarose gel electrophoresis. cDNA was transcribed from 1 μg total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a thermal cycler (PTC200, MJ Research). Expression of target genes and the endogenous control, GAPDH, was assessed by real-time qPCR using the TaqMan® Gene Expression Assays (summarized in **Table 1**) and StepOnePlus™ Real-Time PCR System (Applied Biosystems) according to manufacturer's recommendations. Reactions with no templates and RNA control were included as negative controls. Relative quantification of gene expression was normalized to the housekeeping gene GAPDH, using the $2^{-\Delta\Delta ct}$ method, and illustrated as fold change (Livak and Schmittgen, 2001).

Statistical Analysis

Experimental data from at least four independent experiments were analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, United States). Values are presented as box-and-whiskers plot (box from 25th to 75th percentile and whisker min to max). Comparisons between experimental groups were made using one-way ANOVA followed by the Tukey post-test for multiple comparisons and $p < 0.05$ was considered to be significant.

RESULTS

Cyclosporin A is an immunosuppressant medication that has been reported to exhibit neuroprotective properties in multiple experimental models (30–32). Therefore, we investigated the potential neuroprotective effect of pre-treatment with CsA (1 and 10 μM) in a simulated ischemia/reperfusion-induced injury model (1 h OGD followed by 24 h restoration of oxygen,

TABLE 1 | TaqMan® Gene Expression Assays.

Gene	Assay ID
GAPDH	99999915_g1
Iba1	00479862_g1
IL-1β	00434228_m1
IL-1α	00439620_m1
IL-6	00446190_m1
iNos	00440502_m1
MCP1 (Ccl2)	00441242_m1
TGF-β1	01178820_m1
TNF-α	00443260_g1

glucose, and serum) in either a monoculture of murine primary neurons (DIV7) prepared from E15 mice, BV-2 microglia, or in an OHSC (DIV14) prepared from early postnatal (P3–5) mice that retains the cytoarchitecture and synaptic circuits of the hippocampus. Additionally, moderate hypothermia (33.5°C) was applied post-OGD to both, the *in vitro* and *ex vivo* models, to investigate any potential additive neuroprotective effects of the combined treatment.

Cytotoxicity in Primary Neuron and Microglia Monocultures

We observed that initial exposure to OGD for 1 h did not significantly induce necrotic cell death compared to normoxic control in the primary neuronal and BV-2 microglial cell cultures, as measured by LDH release (**Figures 2A,B**). However, treatment with 10 μM CsA under both normoxic and OGD conditions resulted in measurable toxicity in the primary neurons, as observed by significantly increased LDH release compared to Normoxia control (2.8-fold and 4.1-fold increase, respectively), while 1 μM CsA was not toxic (**Figure 2A**). Furthermore, BV-2 microglia were resistant to CsA-induced toxicity, as no significant increases in LDH releases were measurable after exposure to 1 h OGD and treatment with 1 and 10 μM CsA (**Figure 2B**).

Exposure to 1 h OGD followed by 24 h reperfusion (OGD/R) resulted in a significantly higher LDH release (4.4-fold increase relative to Normoxia/R control) in the primary neurons (**Figure 2C**). Treatment with 1 and 10 μM CsA had no additive cytotoxic effect and necrotic cell death was not attenuated by cooling. In contrast, BV-2 microglia exposed to OGD/R showed no observable necrotic cell death, but treatment with CsA was observed to be toxic in a dose dependent manner under both Normoxia/R and OGD/R conditions, as measurable by an incremental increase in LDH release that was significant with 10 μM CsA (Normoxia/R: 4.0-fold; OGD/R: 3.8-fold) (**Figure 2D**). Treatment with hypothermia resulted in only slight decreases in LDH releases that did not reach significancy under all test conditions.

Interestingly, longer exposure to CsA [1 h (**Figure 2B**) vs. 1+24 h (**Figure 2D**)] induced higher LDH release in BV-2 microglia, which was not observable in the neuronal cultures. BV-2 exposed to 1 μM CsA under normoxic conditions resulted in an increase of LDH release from 1.6-fold to 3.0-fold, and 10 μM CsA resulted in an increase from 1.9-fold to 4.0-fold, respectively (**Figures 2B,D**).

Cytotoxicity in Organotypic Hippocampal Slice Cultures

Organotypic hippocampal slice culture are a suitable model for studying cell death and neuroprotective agents *ex vivo*, as the complex cellular structures and cell interactions are preserved. Therefore, we further investigated the cytotoxic effects of exposure to 1 h OGD and treatment with 10 μM CsA in OHSCs prepared from early postnatal (P3–5) mice and cultivated for 14 days (DIV14). Necrotic cell death was

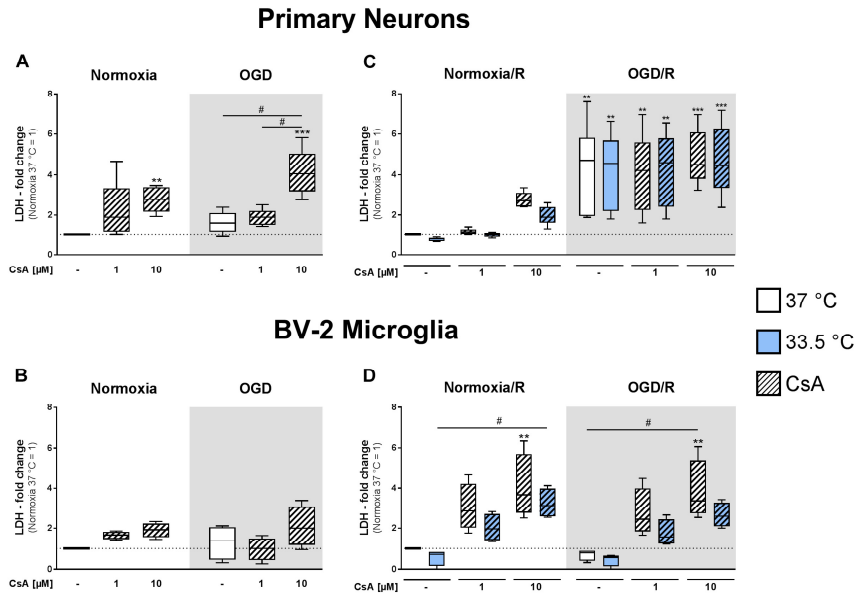

FIGURE 2 | Lactate dehydrogenase releases were measured from primary neurons **(A)** and BV-2 microglia cells **(B)** in the experimental control group exposed to 1 h normoxia (21% O_2 in glucose-containing medium) and simulated ischemia group exposed to 1 h oxygen-glucose deprivation (OGD at 0.2% O_2 in glucose-depleted medium) at 37°C, and also after reperfusion from primary neurons **(C)** and BV-2 microglia cells **(D)** exposed to 1 h normoxia + 24 h reperfusion with complete medium (Normoxia/R) or 1 h OGD + 24 h reperfusion (OGD/R) at either 37 or 33.5°C. All CsA groups were pre-treated 1 h before experimental start with either 1 or 10 μM CsA at 37°C. Data from 4 to 6 individual experiments are presented as box-and-whiskers plot (box from 25th to 75th percentile and whisker min to max). Statistical analysis were conducted using one-way ANOVA followed by the Tukey *post hoc* test; $^{**}p < 0.01$, $^{***}p < 0.001$ compared to Normoxia at 37°C, and $^{\#}p < 0.05$ for group comparison were considered significant.

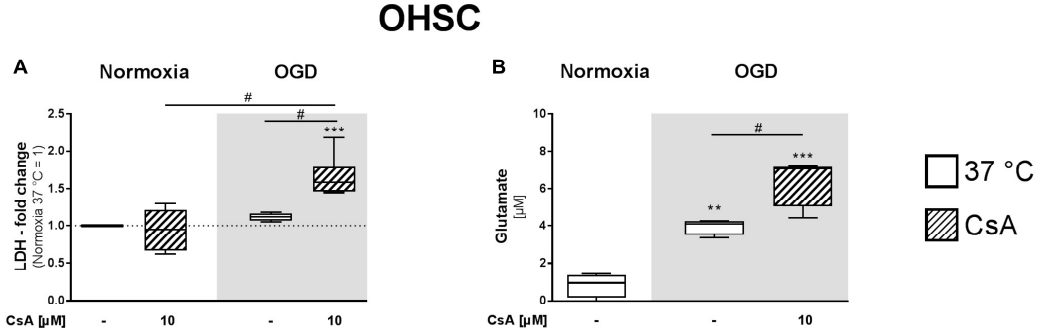

FIGURE 3 | (A) LDH and **(B)** glutamate releases from OHSCs were assessed from the cultured supernatants of the experimental control group exposed to 1 h normoxia (21% O_2 in glucose-containing medium) and simulated ischemia group exposed to 1 h oxygen-glucose deprivation (OGD at 0.2% O_2 in glucose-depleted medium) at 37°C. All CsA containing groups were pre-treated 1 h before experimental start with 10 μM CsA at 37°C. Data from 4 to 5 individual experiments are presented as box-and-whiskers plot (box from 25th to 75th percentile and whisker min to max). Statistical analysis were conducted using one-way ANOVA followed by the Tukey *post hoc* test; $^{**}p < 0.01$, $^{***}p < 0.001$ compared to Normoxia at 37°C, and $^{\#}p < 0.05$ for group comparison were considered significant.

assessed by the release of LDH and glutamate, a neuronal excitotoxicity marker. We did not observe a significant increase in LDH release after 1 h exposure to OGD or treatment with 10 μM CsA under normoxic condition alone. However, treatment with 10 μM CsA under OGD condition resulted in an increase in LDH release that was significantly higher than for both treatments with normoxic CsA and OGD alone (**Figure 3A**). Interestingly, we observed significantly higher glutamate release in the OHSC exposed to 1 h OGD compared to the normoxic control (3.9 vs. 0.8 μM, respectively) (**Figure 3B**). Significantly higher release of glutamate was also observed in OHSC treated with 10 μM CsA under OGD condition compared to normoxic control (6.42 vs. 0.83 μM, respectively), confirming our LDH assessment indicating necrosis in the OHSCs.

Due to high serum concentration in the reperfusion medium, LDH and glutamate levels were not detectable after OGD/R-induced injury. Therefore, we performed PI staining to assess necrotic cell death in the OHSC after reperfusion (**Figure 4**). Exposure of the OHSCs to OGD/R at 37°C resulted in a marked increase in PI positive cells, as compared to Normoxia/R at 37°C. OHSCs exposed to OGD/R at 33.5°C resulted in less observable PI positive cells. OHSCs exposed to Normoxia/R +10 μM CsA at 37°C also showed a marginal increased in PI positive cells that dramatically increased when CsA was applied in combination with OGD/R at 37°C. No observable differences in PI positive cells were seen between OGD/R and OGD/R+CsA slices incubated at 37°C, nor with OGD/R+CsA slices incubated at 33.5°C.

Release of DAMPs

Soluble inflammatory mediators are released from the necrotic brain tissue. Therefore, we isolated extracellular proteins from cell-cultured supernatants to investigate the release of inflammation inducing DAMPs from primary neurons and BV-2 microglia exposed to 1 h OGD. Correlating with the LDH measurements, western blot analysis showed significantly higher releases of HMGB1 and HSP70 in primary neurons treated with 10 μM CsA under OGD conditions, in comparison to normoxic control, as well as to all other OGD treated groups (**Figures 5A**,**B**). Additionally, we observed a trend toward a higher release of CIRBP in neurons treated with 10 μM CsA under OGD conditions (**Figure 5C**). Also in correlation to LDH release, no significant increases in extracellular HMGB1 and HSP70 were observed in non-necrotic BV-2 microglia exposed to OGD and treated with 10 μM CsA (**Figures 5D**,**E**). Interestingly, we observed elevated but non-significant DAMPs releases in all OGD damaged BV-2 cells in comparison to Normoxia control, which was not seen in the primary neurons. Extracellular CIRBP was not detectable in the BV-2 microglia cultured supernatant. Furthermore, none of the investigated DAMPs were detectable in the cultured supernatant of the OHSCs.

Cytokine and Chemokine Expression in BV-2 Microglia, Primary Neurons, and OHSC

Based on our observation of increased DAMPs release from necrotic primary neurons, we next investigated the inflammatory response in the immune competent BV-2 microglia, in primary neurons, and OHSC after exposure to OGD/R and treatment with CsA where necrosis was detectable in neurons and OHSC. Additionally, we also investigated the effect of cooling (33.5°C) applied during the reperfusion phase on inflammatory cytokines and chemokines gene expressions.

Cytokine Expressions in BV-2 Microglia and Primary Neurons

We observed a significant increase in IL-6 expression in the BV-2 microglia treated with 10 μM CsA under both Normoxia/R and OGD/R conditions, which correlated with the observed necrotic cell death (**Figure 6A**). Cooling significantly reduced IL-6 expression in CsA treated Normoxia/R control and in weaker extend in the OGD/R injury group. Similar increased IL-6 expression was also observed in the primary

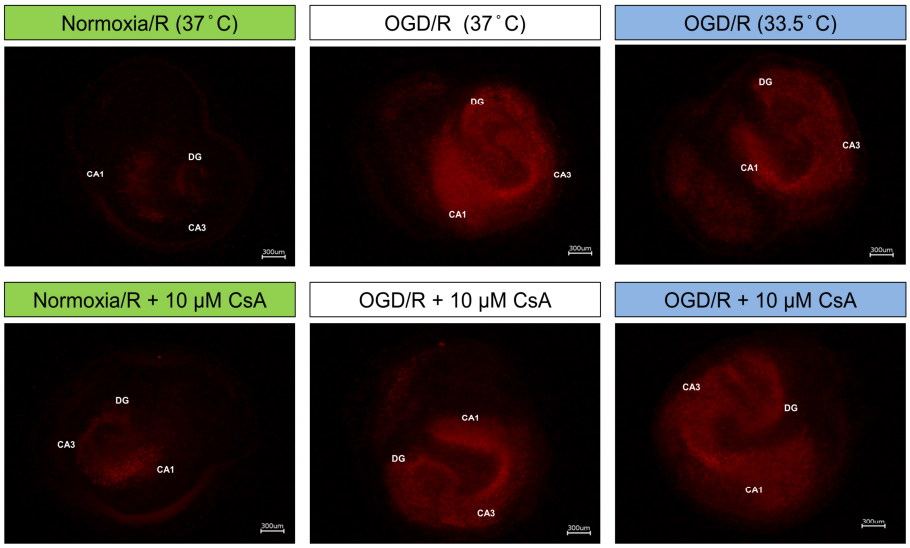

FIGURE 4 | Propidium iodide (PI) staining; OHSCs in the experimental control group (Normoxia/R) were exposed to 1 h normoxia (21% O_2 in glucose-containing medium) + 24 h reperfusion (21% O_2 in glucose-containing medium) and simulated ischemia groups were exposed to 1 h oxygen-glucose deprivation (OGD at 0.2% O_2 in glucose-depleted medium) + 24 h reperfusion (OGD/R at 21% O_2 in glucose-containing medium) at either 37 or 33.5°C. All cyclosporin A (CsA) containing groups were pre-treated 1 h before experimental start with 10 μM CsA at 37°C and maintained throughout the duration of the experiment. Images are shown at 40× magnification. CA, Cornu Ammonis; DG, Dentate Gyrus.

neurons treated with 10 μM CsA (**Figure 6I**). Interestingly, TNF-α expression was significantly induced by cooling under both Normoxia/R and OGD/R conditions in BV-2 microglia, which was attenuated by treatment with CsA (**Figure 6B**). Additionally, cooling and CsA alone and in combination significantly inhibited IL-1β expression under both Normoxia/R and OGD/R conditions (**Figure 6C**), but had no significant effect on IL-1α expression in BV-2 microglia (**Figure 6D**). TNF-α, IL-1β, and IL-1α expressions were under detection limit in the primary neurons.

Cytokine Expressions in the OHSC Model

We next investigated if exposure to OGD/R injury and treatment with CsA had a similar cytokine expression regulation in the OHSC model as observed in the BV-2 microglia. In general, inflammatory cytokines expressions in the OHSC model did not reflect what we observed in the BV-2 microglia monoculture. Interestingly, the combined treatment of 10 μM CsA, OGD/R, and cooling resulted in the greatest general increase in cytokines expressions. Under these combined conditions, IL-6 expression was significantly higher than Normoxia/R control and OGD/R+CsA, indicating a profound effect of cooling on IL-6 expression (**Figure 6E**). TNF-α expression was significantly higher than Normoxia/R control in the OGD/R+CsA treated group with no observable effect of cooling (**Figure 6F**). IL-1β expression was not suppressed as observed in the BV-2 microglia (**Figure 6G**), and IL-1α expression appears to be more induced by cooling under both OGD/R and OGD/R+CsA treatments compared to 37°C Normoxia/R group (**Figure 6H**).

Chemokine and Growth Factor Expressions in BV-2 Microglia and Primary Neurons

Furthermore, we investigated the expression of targets regulating migration and activation of microglia and monocytes. In BV-2 microglia we observed a trend toward hypothermia-induced MCP1 expression, whereas treatment with CsA rather reduced MCP1 expression in comparison to Normoxia/R control (**Figure 7A**). In contrast to BV-2 microglia, we found an increased MCP1 expression in primary neurons treated with 10 μM CsA, which was even higher at 33.5°C in the OGD/R injured group (**Figure 7I**).

Activation of microglia is associated with increased Iba1 expression (Hovens et al., 2014). Similar to MCP1, we found Iba1 expression significantly induced in the cooled OGD/R injured group, while treatment with CsA significantly decreased Iba1 expression (**Figure 7B**). The expression of growth factor TGF-β1 was unchanged in all BV-2 microglia groups (**Figure 7C**). Additionally, we found iNOS expression to be significantly suppressed by cooling under Normoxia/R, and to a less extend by OGD/R at 33.5°C and by CsA treatment (**Figure 7D**). Iba1, TGF-β1, and iNOS expressions were under detection limit in the primary neurons.

Chemokine and Growth Factor Expressions in OHSC

In the OHSC model we observed a strong increase in chemokine MCP1 expression in OGD/R and OGD/R+CsA compared to Normoxia/R control (**Figure 7E**). A similar response

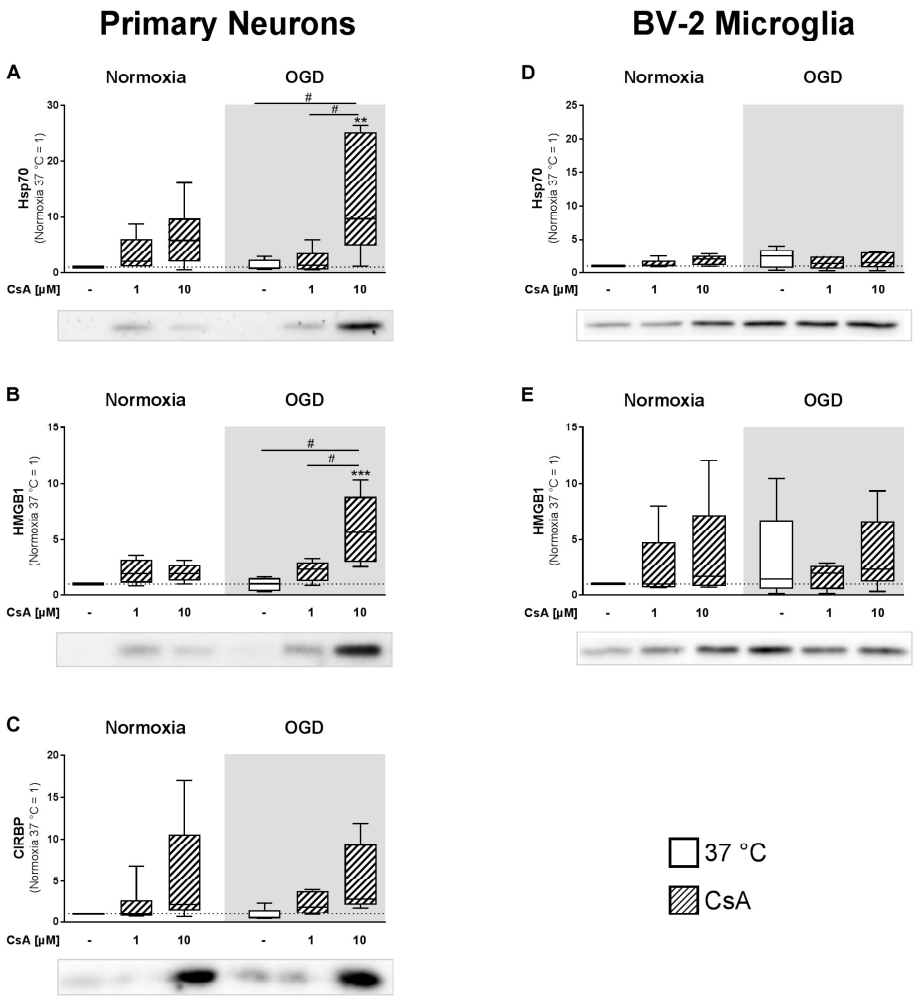

FIGURE 5 | Western Blot analysis was used to assess extracellular DAMPs, **(A,D)** heat shock protein 70 (Hsp70), **(B,E)** high mobility group box 1 (HMGB1), and **(C)** cold-inducible RNA-binding protein (CIRBP) released into the cultured supernatant from primary neurons and BV-2 microglial cells. The experimental control group was exposed to 1 h normoxia (21% O_2 in glucose-containing medium) and simulated ischemia group was exposed to 1 h oxygen-glucose deprivation (OGD at 0.2% O_2 in glucose-depleted medium) at 37°C. Quantitative densitometric analysis from 5 to 6 individual experiments is presented as box-and-whiskers plot (box from 25th to 75th percentile and whisker min to max), along with the representative immunoblots. Statistical analysis were conducted using one-way ANOVA followed by the Tukey *post hoc* test; $^{**}p < 0.01$, $^{***}p < 0.001$ compared to normoxia at 37°C, and $^{\#}p < 0.05$ for group comparison were considered significant.

was also found in primary neurons (**Figure 7I**). Iba1 gene expression was induced in OGD/R injury alone and in combination with CsA (**Figure 7F**). Comparable to Iba1, the expression of growth factor TGF-β1 was increasingly expressed in OGD/R injured slices, while cooling and CsA treatment had no additional effect (**Figure 7G**). Interestingly, iNOS expression was significantly decreased by OGD/R-induced injury and increased to Normoxia/R level by OGD/R+CsA (**Figure 7H**).

DISCUSSION

Neuroinflammation plays a central role in the pathogenesis of ischemic brain injury (Bhalala et al., 2014; Hagberg et al., 2015). Therefore, we evaluated the anti-inflammatory and neuroprotective effect of clinically discussed hypothermia in combination with CsA immunosuppressant treatment on OGD/R injured brain cells. In our study treatment with hypothermia and CsA caused an alteration in the inflammatory pathways. While pro-inflammatory IL-1β and iNOS expressions are effectually suppressed in BV-2 microglia and OHSC, expression of TNF-α, IL-1α, MCP1, Iba1, and TGF-β1 are increased in OGD/R injured OHSC. We found that primary neurons undergo cell death by OGD/R-induced injury and by CsA treatment, suggesting a complex activation of inflammatory pathways through the involvement of a secondary damage mechanism in complex slice culture. Our findings demonstrate that CsA, a highly specific inhibitor

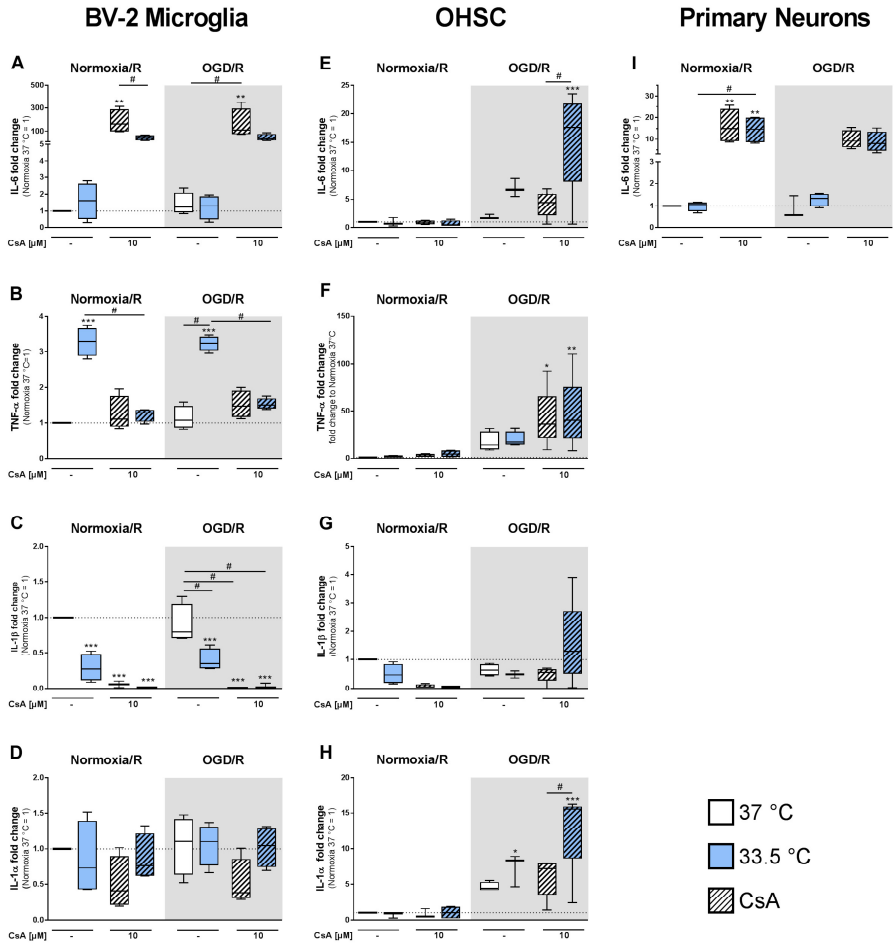

FIGURE 6 | RT-qPCR was used to assess gene expressions in BV-2 microglia, OHSCs, and primary neurons for inflammatory **(A,E,I)** IL-6, **(B,F)** TNF-α, **(C,G)** IL-1β, and **(D,H)** IL-1α after exposure to Normoxia/R (1 h normoxia + 24 h reperfusion at 21% O_2 in glucose-containing medium) or OGD/R (1 h OGD + 24 h reperfusion at 0.2% O_2 in glucose-depleted medium) at either 37 or 33.5°C. All CsA containing groups were pre-treated with 10 μM CsA at 37°C. Data from 4 to 5 individual experiments are presented as box-and-whiskers plot (box from 25th to 75th percentile and whisker min to max). Statistical analysis were conducted using one-way ANOVA followed by the Tukey *post hoc* test; $^{*}p < 0.01$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to Normoxia/R at 37°C, and $^{\#}p < 0.05$ for group comparison were considered significant.

of calcineurin, affects the survival of primary neurons and modulate the inflammatory response in microglial cultures, as well as in the OHSC.

Cytotoxicity-Induced DAMPs Release

Primary neurons pre-treated with 10 μM CsA followed by 1 h exposure to OGD undergo necrotic cell death, as indicated by increased LDH release. Additionally, increased amounts of DAMPs, namely Hsp70, HMGB1, and CIRBP were detected in the cultured supernatants. Hsp70 and HMGB1 are classical DAMPs and have been extensively investigated (Asea et al., 2000; Muhammad et al., 2008), whereas the inflammation-inducing DAMP property of extracellular CIRBP is a recent observation that has been shown to perpetuate the inflammatory response in hemorrhagic shock and sepsis patients (Qiang et al., 2013). Although both, intracellular Hsp70 and CIRBP, have been shown to have desirable anti-apoptotic properties, their presence in the extracellular matrix has been shown to initiate inflammatory responses (Liao et al., 2017; Kim et al., 2018). Their affinity to bind to surface receptors, including RAGE, toll-like receptor 2 (TLR2), and 4 (TLR4) on adjacent cells leads to the activation of the nuclear factor "kappa-light-chain-enhancer" of activated B-cells (NF-κB) pathway and transcription of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Johnson and Fleshner, 2006; Andersson and Tracey, 2011; Zhou et al., 2014). In comparison to neurons, BV-2 microglia were more vulnerable to OGD-induced release of HMGB1 and Hsp70, which did not reach significance, but were less vulnerable to OGD+CsA-induced cytotoxicity, which resulted in no additional DAMPs release.

Organotypic hippocampal slice culture exposed to 1 h of OGD had a significant increased release of glutamate, but no increase in LDH release was measureable. Under

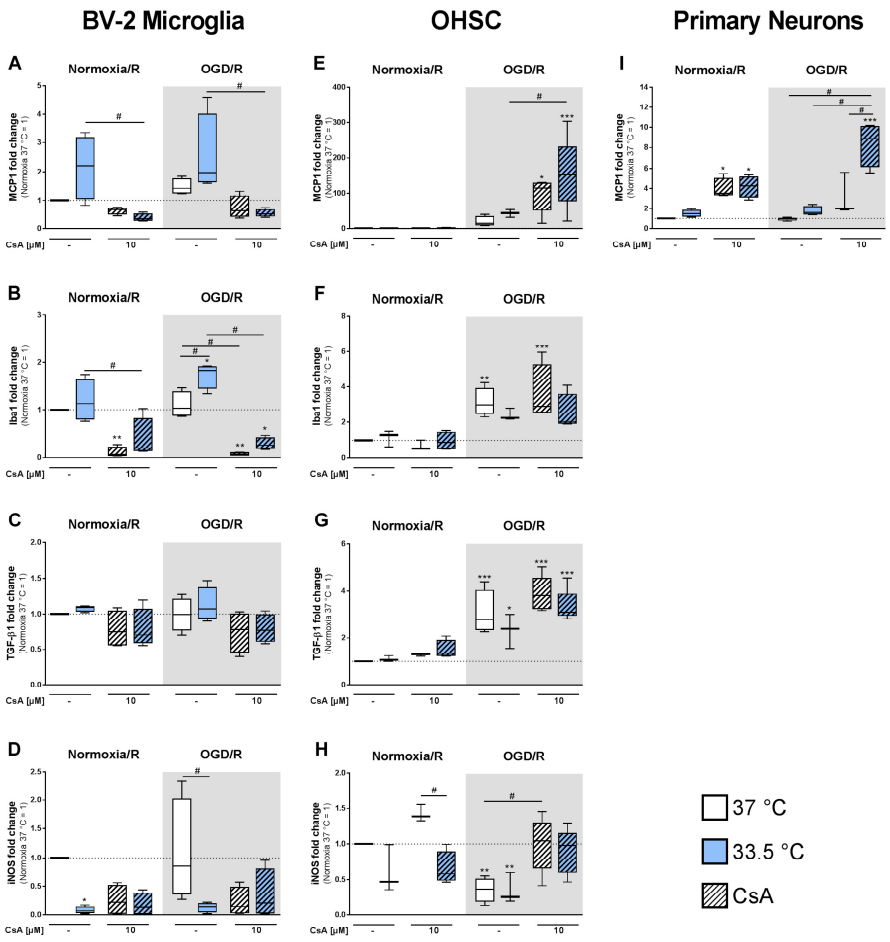

FIGURE 7 | RT-qPCR was used to assess gene expressions in the BV-2 microglia, OHSCs, and primary neurons for **(A,E,I)** MCP1, **(B,F)** Iba1, **(C,G)** TGF-β1, and **(D,H)** iNOS after exposure to Normoxia/R (1 h normoxia + 24 h reperfusion at 21% O_2 in glucose-containing medium) or OGD/R (1 h OGD + 24 h reperfusion at 0.2% O_2 in glucose-depleted medium) at either 37 or 33.5°C. All CsA containing groups were pre-treated with 10 µM CsA at 37°C. Data from 4 to 5 individual experiments are presented as box-and-whiskers plot (box from 25th to 75th percentile and whisker min to max). Statistical analysis were conducted using one-way ANOVA followed by the Tukey *post hoc* test; $^{*}p < 0.01$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to Normoxia/R at 37°C, and $^{\#}p < 0.05$ for group comparison were considered significant.

non-pathological conditions glutamate in the extracellular space is cleared by astrocytes via glutamate transporters, but under ischemic conditions the uptake is reversed due to ion gradients changes, resulting in the release of glutamate to the extracellular space. High concentration of glutamate can cause extensive neuronal injury and subsequently trigger cell death (Rossi et al., 2000; Nishizawa, 2001; Zhang et al., 2019). The addition of CsA during the OGD phase resulted in an increase in LDH release and an additional increase of glutamate in the cultured supernatant, presumably by necrotic cells, as we also observed CsA-induced cell death in both microglial and neuronal mono-cultures. Our findings are in line with an *in vitro* study of primary mixed neurons-astrocytes culture showing CsA-induced cytotoxicity only in the neurons, while the astrocytes were unharmed (Kaminska et al., 2001).

Reperfusion-induced injury occurs when restoration of energy and oxygen after an OGD phase is achieved, causing alterations in multiple pathways affecting inflammation, redox-system, and many other signaling pathways (Lopez-Neblina et al., 2005; Mizuma and Yenari, 2017). In line with previous findings describing a higher resistance of microglia than neurons to OGD/R-induced injury, we also observed a marked increase in necrosis in primary neurons but absent in BV-2 microglia after exposure to OGD/R (Goldberg and Choi, 1993; Lim et al., 2006; Li et al., 2007). Surprisingly, neither treatment with moderate hypothermia for 24 h, nor CsA protected the neurons from OGD/R-induced cell death. Conversely, treatment with CsA resulted in BV-2 microglia cell death in a concentration dependent manner, which could be partially attenuated by cooling. In the *ex vivo* OHSCs, we observed a dramatic increase in PI positive cells after exposure to OGD/R at 37°C,

which was decreased by cooling to 33.5°C. Similar exposure to OGD/R at 37°C also resulted in significant cell death in the primary neuronal culture. However, treatment with CsA during OGD/R had no observable protective effect in the slice cultures independent of temperature, as similar intensities in PI staining were also observed in the slices exposed to OGD/R alone. This is in contrast to Liu et al. (2016a), who reported a protective effect of CsA on the mitochondria which was augmented by hypothermia in an *in vivo* rat cardiac arrest model. Unfortunately, quantification of DAMPs release in the reperfusion phase was not possible by western blot analysis due to the high serum concentration in the cultured medium, and warrants further investigation.

Hypothermia

To date, it is widely accepted that multiple pathways and mechanisms are involved in the protective effect of hypothermia (Kurisu and Yenari, 2018). Preclinical studies *in vitro* and *in vivo* suggest that anti-inflammatory mechanisms induced by hypothermia play an important role (Lee et al., 2016). Exposure of BV-2 microglia to hypothermia for 24 h had variable effects on known pro-inflammatory mediators, resulting in increased TNF-α and MCP1 expressions and decreased IL-1β and iNOS expressions under both Normoxia/R and OGD/R conditions. While increased iNOS and IL-1β levels are generally associated with neurotoxic effects, TNF-α also exhibits neuroprotective properties (Kawabori and Yenari, 2015). Lambertsen et al. (2009) showed in an *in vivo* cerebral ischemia model that microglia derived TNF-α enhances neuronal survival. Other *ex vivo* OHSC studies showed that deep hypothermia induces TNF-α secretion, resulting in neurite outgrowths (Schmitt et al., 2010). Nevertheless, neurotoxic effects by high levels of TNF-α in the ischemic brain injury are also well documented (Sriram and O'Callaghan, 2007). Also the expression of the chemokines MCP1 leads to recruitment and migration of leukocyte from the periphery to the site of injury. MCP1 is upregulated in models of cerebral ischemia and inhibition of MCP1 can decrease brain injury (Chen et al., 2003).

When the same OGD/R injury protocol was applied to the OHSC model *ex vivo*, neither significant induction of TNF-α and MCP1 expressions, nor a significant inhibition of IL-1β by cooling was observed. The heterogeneity of cells in the OHSC, with 5–15% of microglial cells in the hippocampus (Lawson et al., 1990) may explain the variability.

Cyclosporin A

Cyclosporin A is a cyclophilin binding substance whose primary immunosuppressive function is to inhibit calcineurin, a Ca^{2+}/calmodulin dependent protein phosphatase. Calcineurin is ubiquitously expressed in most tissues, but at particularly high concentrations in the brain. In glia cells calcineurin plays a global role in neuroinflammation, as it interacts and modulates multiple transcription factors, including NFAT, NF-κB, and AP1, which are associated with cytokines expression (Furman and Norris, 2014).

In the BV-2 microglia, cooling suppressed IL-1β and iNOS expressions, but also induced TNF-α and MCP1 expressions, which was attenuated by CsA treatment. Increased pro-inflammatory iNOS expression has been observed to result in the production of high amounts of NO, which attributes to brain cytotoxicity and promotes ischemic cell death (Amantea et al., 2009; Terpolilli et al., 2012). Zawadzka et al. (2012) observed that CsA inhibits iNOS expression in an *in vitro* model of lipopolysaccharide stimulated microglia, by interfering with the MAPK and NF-κB signaling pathways. Our findings indicate that NF-κB driven cytokines expressions are also inhibited by CsA treatment. This is not surprising as calcineurin has been shown to be indirectly involved in the activation of NF-κB by degrading IκBα, as well as NFATs regulated cytokine gene expressions by coupling with AP1 or NF-κB, amongst others (Macian et al., 2001; Sama et al., 2008; Palkowitsch et al., 2011). Multiple isoforms of NFAT that are calcineurin activated transcription factors have been shown to be expressed by microglia and can be specifically inhibited by the NFAT inhibitor VIVIT, resulting in decreased secretions of TNF-α and MCP1 (Nagamoto-Combs and Combs, 2010; Rojanathammanee et al., 2015). Additionally, microglia activation marker, Iba1, expression was also inhibited by CsA. In contrast to reduced MCP1 expression observed in the BV-2 microglia, CsA induces expression of MCP1 in the primary neuronal cultures under both Normoxia/R and OGD/R conditions. This is not surprising, as neurons have been observed to be capable of expressing chemokines, including MCP1, during the early phase after ischemia in an *in vivo* model of focal cerebral ischemia (Che et al., 2001).

Interestingly, treatment with CsA upregulated IL-6 expression, but had no measurable effect on TGF-β1 and IL-1α expressions in the BV-2 microglia. IL-6 was also the only cytokine we observed to be expressed under OGD/R+CsA treatment in primary neurons. IL-6 can be secreted by both immune (microglia) and non-immune (neurons) cells and function as a neurotrophic factor and inhibitor of neuronal death (Loddick et al., 1998). At the same time IL-6 is involved in the pathological progression of several inflammatory diseases (Rothaug et al., 2016).

Altogether, CsA exhibits an immunosuppressive effect in a BV-2 microglia model, but it also had a toxic effect at the same concentration in a murine dissociated neuronal culture. The response to OGD/R-induced injury in combination with CsA and hypothermia treatment was also investigated in a more complex *ex vivo* OHSC model, which shows a different pattern of response to simulated ischemia than the individual primary neuronal cultures and BV-2 microglia. We did not observe significant increased induction of the inflammatory pathways investigated in the OGD/R-induced injured slices, but surprisingly increases in TNF-α, IL-1α, IL-6, and MCP1 expressions were observed when 10 μM CsA was additionally introduced, which was not observed in the Normoxia/R control treated with CsA nor in the BV-2 microglia under OGD/R+CsA conditions. As astrocytes are the most abundant glial cell type in the brain capable of participating in the immune response (Becerra-Calixto and Cardona-Gomez, 2017), the observed increase in inflammatory response in our *ex vivo* OHSC model could be considered to be astrocytes driven. Increased MCP1 expression has been observed in hippocampal astrocytes after *in vivo* transient global

ischemia (Sakurai-Yamashita et al., 2006), which can trigger the adaptive immunity response in the inflamed CNS (Farina et al., 2007). Additionally, *in vitro* studies with primary astrocytes showed longer exposure to OGD (24 h) resulted in increased TNF-α and IL-1β secretions, which was attenuated by CsA treatment (Gabryel et al., 2004). Moreover, *in vivo* studies of transient middle cerebral artery occlusion in a rat model found decreased TNF-α secretion after treatment with CsA containing nanoliposomes compared to non-treated animals (Partoazar et al., 2017). This is in contrast to our findings in the OHSC treated with OGD/R+CsA, indicating that the putative protective effect of CsA may be dominated by or overlaid with other pro-inflammatory mediators.

Other studies showed DAMPs released from necrotic neurons in hippocampal slices or treated with exogenous HMGB1 lead to induced expression of inflammatory cytokines TNF-α and IL-1β via TLR4 activation in microglia cells (Zou and Crews, 2014). As we found LDH and glutamate secretions are increased in OGD+CsA-induced damaged OHSC, it is likely that various DAMPs are released from necrotic neurons, thereby activating neighboring microglia and astrocytes to drive the secondary inflammatory process after OGD/R-induced injury. While CsA mediated suppression of microglia activation, it is not sufficient to reduce the overall inflammatory response in the complex OHSC. To our knowledge, the effect of CsA on the inflammatory response in OGD/R-induced OHSC injury has not been investigated, as most research studies have investigated the amelioration of cell death by CsA treatment, and protection of the mitochondria from OGD/R-induced opening of the mitochondrial permeability transition pores (Kawakami, 2013; Trumbeckaite et al., 2013; Yu et al., 2013).

An inflammatory response in the brain can be both beneficial and harmful, depending on the type and amount of cytokines expressed. An increase in Iba1 expression is associated with microglia activation (Hovens et al., 2014). TGF-β1 is mainly expressed by these activated microglial cells and is associated with a reduction of neuronal cell death and decreased infarct size after cerebral ischemia (Meyers and Kessler, 2017). We observed both Iba1 and TGF-β1 expressions to be increased in OGD/R injured slices, whereas other cytokines were not significantly altered. Our findings indicate a beneficial activation of inflammatory processes after exposure to OGD/R in the OHSC model, but it becomes highly neuroinflammatory when CsA is additionally applied.

We acknowledge several limitations of our study. First, we only assessed cytokine and chemokine mRNA expressions and not the secreted form. Therefore, any conclusion concerning inflammation should be approached with caution, as intracellular and secreted levels may differ. Second, quantifiable necrosis in the hippocampal slice cultures as well as any subsequent DAMPs release after reperfusion was not possible due to high serum concentrations in the cultured media. Therefore, further studies are needed to investigate the impact of DAMPs associated sterile inflammation in CNS cells. Finally, our protocol focused on ischemia sensitive neurons and immunocompetent microglia. As astrocytes constitute a large population of cells in the brain and have an important role in neuroprotection, further studies investigating the effects of CsA and cooling on OGD/R-induced injury in astrocytes are warranted.

CONCLUSION

We found CsA treatment to be effective in suppressing inflammation in a pure microglia culture after OGD/R-induced injury, but causes necrotic cell death in primary neurons. In the complex *ex vivo* slice culture CsA treatment lead to an exacerbated immune response, which was not diminished by hypothermia but instead potentiated an additive effect leading to an increase in neuroinflammation. Based on our findings, the combination of cooling and CsA treatment can hereby not be considered as neuroprotective. In contrast to other studies describing the neuroprotective effects of CsA and hypothermia, we observed the induction of neuroinflammation to the combined treatment in a complex OHSC model.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the guidelines of the Charité – Universitätsmedizin Berlin, Germany and the national ethic principles (registration no. T0044/08).

AUTHOR CONTRIBUTIONS

SW, GT, and KS designed the experiments. SW conducted the experiments and analyzed the data. SW, GT, KS, NR, JK, and FB participated in the discussion of the results. SW prepared the manuscript. GT, JK, NR, and KS reviewed the manuscript. All authors read and approved the final manuscript.

FUNDING

This study was financially supported by the Stiftung KinderHerz, Essen, Germany (Grant Number 2511-3-14-015). We acknowledge support from the German Research Foundation (DFG) and the Open Access Publication Fund of Charité – Universitätsmedizin Berlin.

ACKNOWLEDGMENTS

We would like to thank Nora Künzel for her excellent technical assistance.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.2 Publikation 2 (Rosenthal et al. 2017)

Rosenthal LM, Tong G, Walker C, Wowro SJ, Krech J, Pfitzer C, Justus G, Berger F, Schmitt KRL.

„Neuroprotection via RNA-binding protein RBM3 expression is regulated by hypothermia but not by hypoxia in human SK-N-SH neurons."

Hypoxia, 05/ 2017, 5:33-43.

Impact Factor: N/A

Hypoxia

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ORIGINAL RESEARCH

Neuroprotection via RNA-binding protein RBM3 expression is regulated by hypothermia but not by hypoxia in human SK-N-SH neurons

This article was published in the following Dove Press journal:
Hypoxia
23 May 2017
Number of times this article has been viewed

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Objective: Therapeutic hypothermia is an established treatment for perinatal asphyxia. Yet, many term infants continue to die or suffer from neurodevelopmental disability. Several experimental studies have demonstrated a beneficial effect of mild-to-moderate hypothermia after hypoxic injury, but the understanding of hypothermia-induced neuroprotection remains incomplete. In general, global protein synthesis is attenuated by hypothermia, but a small group of RNA-binding proteins including the RNA-binding motif 3 (RBM3) is upregulated in response to cooling. The aim of this study was to establish an in vitro model to investigate the effects of hypoxia and hypothermia on neuronal cell survival, as well as to examine the kinetics of concurrent cold-shock protein RBM3 gene expression.

Methods: Experiments were performed by using human SK-N-SH neurons exposed to different oxygen concentrations (21%, 8%, or 0.2% O_2) for 24 hours followed by moderate hypothermia (33.5°C) or normothermia for 24, 48, or 72 hours. Cell death was determined by quantification of lactate dehydrogenase and neuron-specific enolase releases into the cell cultured medium, and cell morphology was assessed by using immunofluorescence staining. The regulation of RBM3 gene expression was assessed by reverse transcriptase-quantitative polymerase chain reaction and Western blot analysis.

Results: Exposure to hypoxia (0.2% O_2) for 24 hours resulted in significantly increased cell death in SK-N-SH neurons, whereas exposure to 8% O_2 had no significant impact on cell viability. Post-hypoxia treatment with moderate hypothermia for 48 or 72 hours rescued the neurons from hypoxia-induced cell death. Moreover, exposure to severe hypoxia led to observable cell swelling, which was also attenuated by moderate hypothermia. Finally, moderate hypothermia but not hypoxia led to the induction of RBM3 expression on both transcriptional and translational levels.

Conclusion: Moderate hypothermia protects neurons from hypoxia-induced cell death. The expression of the cold-shock protein RBM3 is induced by moderate hypothermia and could be one possible mediator of hypothermia-induced neuroprotection.

Keywords: RBM3, cold-inducible RNA-binding protein, hypothermia, neuroprotection, hypoxia

Introduction

Perinatal asphyxia is the third most frequent cause of death during the neonatal period and a major cause of long-term neurodevelopmental disability in Western industrialized nations.[1] It is a medical condition under which the neonatal brain suffers from oxygen deprivation, which leads to hypoxic neuronal damage.[2] The only evidence-based therapy to prevent neuronal damage due to perinatal asphyxia is to lower the infant's body temperature to 33°C–34°C for 72 hours, as observed in several multicenter randomized controlled trials, to improve survival rates and neurological outcome in term infants.[3–5] Moderate therapeutic hypothermia (32.0°C–33.9°C) is also recommended

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http://dx.doi.org/10.2147/HP.S132462

for other clinical situations, leading to hypoxic–ischemic brain injury, such as out-of-hospital cardiac arrest.[6,7] Yet, many patients with hypoxic–ischemic brain injury survive with a poor neurological outcome or die. Therefore, it is important to further understand the molecular mechanisms of hypothermia-induced neuroprotection and to identify key targets of hypothermia in order to improve current therapy as well as to develop new intervention strategies.

Although the understanding of hypothermia-induced neuroprotection remains incomplete, many experimental studies have shown that hypothermia is able to mitigate several injury processes in the hypoxic–ischemic brain. Hypothermia reduces cerebral metabolism by ~5%–10% per degree cooling and, therefore, decreases oxygen demand in neurons.[8,9] The accumulation of excitatory amino acids such as glutamate released during hypoxic–ischemic brain injury is diminished by hypothermia,[10,11] as well as decreased the production of free radicals and reactive oxygen species (ROS).[12] Furthermore, hypothermia reduces cell death following hypoxia and attenuates inflammatory responses through the inhibition of proinflammatory cytokine expression.[13,14]

Whereas lowering the body temperature inhibits global protein synthesis, a small group of RNA-binding proteins is induced by mild-to-moderate hypothermia. One of these "cold-shock proteins" is the RNA-binding motif 3 (RBM3). First described by Derry et al, RBM3 consists of 157 amino acids, has a molecular weight of 17 kDa, and has an arginine–glycine-rich domain, called the RNA recognition motif (RRM), to bind RNA.[15,16] RBM3 is ubiquitously expressed in the body, but in a temperature-dependent manner. Exposure to moderate hypothermia (32°C) for 24 hours has been observed to increase RBM3 mRNA expression, whereas exposure to elevated temperatures (39°C) resulted in decreased expression.[17] RBM3 expression was also observed to be enhanced in hippocampal murine slices in response to mild hypothermia (33.5°C) on both the transcriptional and translational levels.[18] Furthermore, RBM3 seems to be participating in the oxygen stress response as it has been observed to be upregulated upon exposure to mild or severe hypoxia (8% or 1% O_2, respectively) in non-neuronal cells by an HIF-1-independent mechanism.[19] RBM3 is also highly expressed in the brain of young rats and mice as well as in areas with high translational rates in cerebral tissue of adult rats.[20]

The molecular and cellular function of RBM3 is thought to be multifarious and remains poorly understood. RBM3 is able to bind to the 60s subunit of ribosomes and increases translation.[21,22] Overexpression of RBM3 reduces apoptosis, whereas RBM3 knockout decreases cell viability and inhibits cell proliferation.[23,24] Peretti et al reported that RBM3 is involved in synapse regeneration in a mouse model of neurodegenerative disease.[25] Cold-shock proteins such as RBM3 could be possible mediators of hypothermia-induced neuroprotection. Therefore, an in vitro model with human SK-N-SH neurons was established to investigate the effects of hypoxia and hypothermia on neuronal cell survival and to examine the regulation of RBM3 gene expression.

Methods

Cell culture

SK-N-SH human neuroblastoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were routinely cultured in Dubecco's Modified Eagle's Medium (DMEM) with 10% heat-inactivated fetal calf serum (FCS), 1 mM sodium pyruvate, and 1% penicillin/streptomycin at 37°C and 5% CO_2 in a fully humidified incubator. Culture medium was changed every 2 days and the cells were passed upon reaching ~80% confluency. Cell culture flasks, culture dishes, and 96-well plates were coated with 0.2% gelatin in phosphate-buffered saline (PBS), and the cells were allowed to settle for 24 hours before the experimental start.

Hypoxia/reoxygenation

Hypoxia was induced in the SK-N-SH neurons by using a three-gas-regulated incubator (Binder, Tuttlingen, Germany) with 0.2% O_2 (hypoxia), or 8% O_2 (subatmospheric oxygen tension), 5% CO_2 and N_2 balance at 37°C for 24 hours, followed by reoxygenation at atmospheric normoxic conditions (95% air and 5% CO_2) for up to an additional 72 hours. Control groups were maintained at atmospheric normoxia (21% O_2) for the duration of the experiment (Figure 1). SK-N-SH neurons were cultured in DMEM with 10% FCS and 1% penicillin/streptomycin.

Moderate therapeutic hypothermia

Post-hypoxia moderate therapeutic hypothermia was subsequently applied by cooling the neurons to 33.5°C for 24, 48, or 72 hours in a CO_2 incubator with 95% air, 5% CO_2, and 95% humidity (Hereaus, Hanau, Germany), in accordance with the current guidelines for treatment of perinatal asphyxia. Normothermic experimental groups were maintained at 37°C (Figure 1).

Immunofluorescent microscopy

To examine cell morphology, SK-N-SH cells were stained for neuron-specific beta III-Tubulin (Covance, Princeton, NJ, USA) and 4′,6-diamidin-2-phenylindol (DAPI; Pierce, Rock-

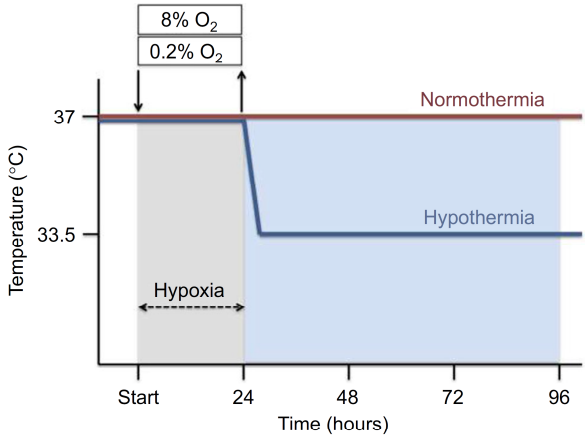

Figure 1 Time temperature protocol. SK-N-SH cells were exposed to either 0.2% or 8% O_2 for 24 hours followed by normothermia (37°C) or hypothermia (33.5°C) for 24, 48 or 72 hours (total 48, 72 and 96 hours).

ford, IL, USA) nuclear counterstain according to an established protocol. Briefly, cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.3% Triton X-100 in PBS, and blocked with 10% goat serum for nonspecific binding. The primary antibody (anti-beta III-Tubulin, 1:2000) was diluted in 0.5% bovine serum albumin in PBS, and cells were incubated overnight at 4°C with gentle agitation. Cells were then washed and incubated with the secondary antibody (Texas Red; Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour at room temperature and protected from light with gentle agitation. Subsequently, cells were washed and then incubated with DAPI (1:2000) for 10 minutes at room temperature and protected from light with gentle agitation. After a final washing with PBS, cover slips were mounted onto glass slides and examined by fluorescent microscopy.

Cell death

For quantification of cell death, the enzymatic activity of lactate dehydrogenase (LDH) released from the cytosol of damaged cells was determined by using a colorimetric Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Extinctions were measured at 570 nm in a microtiter plate reader (Multiscan Ascent; Thermo Fisher Scientific). Optical density values were normalized to the normoxic and normothermic control for each corresponding time point. In addition, release of neuron-specific enolase (NSE) from damaged cells was measured by ELISA (Roche Diagnostics).

ATP concentration

Intracellular ATP levels were quantified by using the CellTiterGlo®Luminescent Cell Viability Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Luminescence intensity was measured with an integrity time of 500 ms.

Quantitative real time polymerase chain reaction analysis

SK-N-SH cells were seeded in 22 cm^2 cell culture dishes at a density of 2×10^5 cells per dish 24 hours before the experimental start. Total cellular RNA extraction was performed by using the peqGOLD RNAPure™ kit (PEQLAB Biotechnology, Erlangen, Germany) according to the manufacturer's instructions. RNA concentration was analyzed employing a NanoDrop spectrophotometer (Thermo Scientific, Karlsruhe, Germany). Two thousand nanograms of total RNA was reversely transcribed into cDNA by using Moloney murine leukemia virus reverse transcriptase and random primer (Promega, Madison, WI, USA) in a reaction mixture including DNAse I treatment. The products of RBM3 and GAPDH as housekeeping gene were quantified in real time by using the following dye-labeled fluorogenic reporter oligonucleotide TaqMan™ probes (Thermo Scientific Fisher): RBM3 Hs00943160_g1 and GAPDH Hs03929097_g1.

Protein extraction and Western blot analysis

SK-N-SH cells were seeded in 60 cm^2 cell culture dishes at cell densities between 1×10^6 and 4×10^6 cells per dish differing for each time point. Cells were allowed to settle 24 hours before experiments. After experiments, SK-N-SH cells were mechanically scratched off the dish surface and lysated by adding a protein lyses buffer ([20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 1:100 of protease inhibitor cocktail]; Sigma-Aldrich, Munich, Germany). Protein concentration was analyzed employing the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Equivalent amounts of protein were loaded on a 12% gel for SDS-PAGE. Immunoblotting to a nitrocellulose membrane (Biostep GmbH, Jahnsdorf, Germany) was performed by using the tank blotting transfer method (Bio-Rad Laboratories GmbH, Munich, Germany). For immunodetection, anti-RBM3 (1:1000, Cat#14363-1-AP; Proteintech, Chicago, IL, USA) and anti-beta-actin (1:10,000, Cat#A5441; Sigma-Aldrich) were used as primary antibodies followed by incubation with anti-rabbit IgG-HRP (1:10,000, Cat#711-035-152, Jackson, ImmunoResearch, West Grove, PA, USA) as secondary antibody. Proteins were visualized by using SuperSignal West Dura Chemiluminescent Substrate according to the manufacturer's instructions (Thermo Fisher

Scientific, Bonn, Germany). Densitometry analysis was performed with Image Lab (Bio-Rad Laboratories GmbH, Munich, Germany).

Statistical analysis

Data were graphed and analyzed with Prism GraphPad 6 (GraphPad Software Inc., Lajolla, CA, USA). All experiments were performed at minimum three times, and results are presented as mean ± SD. Variability between groups was compared by one-way analysis of variance (ANOVA) followed by Bonferroni's post-test for correction of multiple comparisons. Statistical comparison of two non-paired samples was performed by using the Student's *t*-test. For all analyses, $p<0.05$ was considered statistically significant.

Results

LDH

Cell death or cytotoxicity is typically quantified by cellular membrane damage, which results in the release of intracellular proteins such as the LDH into the cultured medium. In the authors' in vitro model, exposure of the SK-N-SH neurons to hypoxia (0.2% O_2 for 24 hours) did not result in a significant increase of LDH release compared to the atmospheric normoxia control directly after exposure (Figure 2A). However, 24 hours after the hypoxic event, LDH release was significantly higher compared to groups maintained at 21% O_2 for the same duration (48 hours after experimental start). Application of moderate hypothermia (33.5°C) resulted in decreased LDH release in comparison to neurons maintained at normothermic conditions after hypoxia, reaching a significant level at 48 and 72 hours of hypothermic treatment (eg, 72 and 96 hours after experimental start). SK-N-SH neurons that were only exposed to hypothermia for 24, 48, or 72 hours at atmospheric normoxia (21% O_2) at all times showed the lowest LDH releases.

Exposure to subatmospheric oxygen tension (8% O_2 for 24 hours) had no observable impact on SK-N-SH neuron viability. The LDH release did not differ from that in neurons under atmospheric normoxia (Figure 2B). However, exposure to moderate hypothermia for 24, 48, or 72 hours significantly decreased LDH release in SK-N-SH neurons that were exposed to 8% O_2 as compared to normothermic controls at 37°C. The amount of LDH release was comparable with that of SK-N-SH treated with moderate hypothermia at 21% O_2.

NSE

NSE is an established biomarker for quantification of neurological damage. In accordance with the LDH data, hypoxia (0.2% O_2 for 24 hours) followed by 72 hours of atmospheric normoxia resulted in a significant increase in NSE release in the SK-N-SH cells, indicating cellular injury (Figure 3). Moderate hypothermia (33.5°C) for 72 hours significantly reduced NSE concentrations and, therefore, attenuated the neurological damage due to hypoxia. Furthermore, NSE concentration in cooled post-hypoxic SK-N-SH was comparable to that in the undamaged control group kept at 21% O_2 and normothermia (37°C) at all observed time points. The release of NSE was significantly lower in SK-N-SH neurons that were only exposed to moderate hypothermia for 72 hours compared to undamaged SK-N-SH at 37°C.

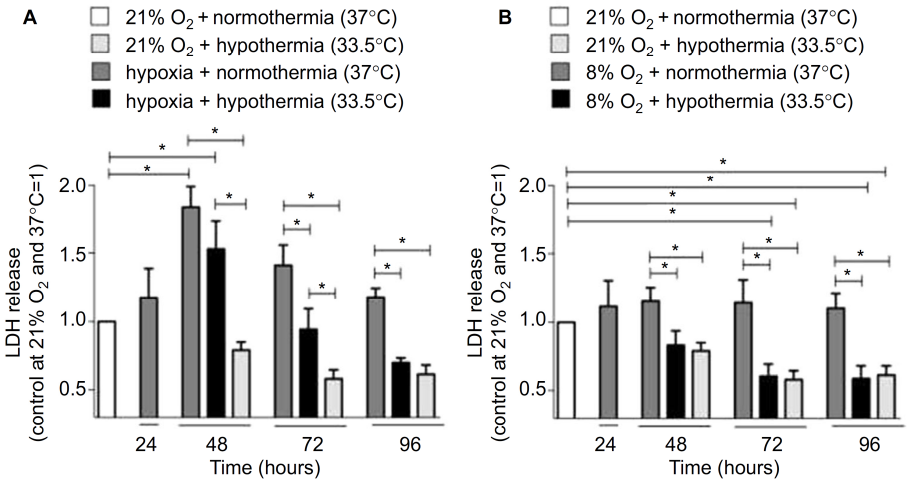

Figure 2 LDH release in SK-N-SH cells exposed to (**A**) 0.2% or (**B**) 8% O_2 for 24 hours followed by normothermia/hypothermia for 24, 48, or 72 hours (total 48, 72 and 96 hours, n=3–4, *$p<0.05$).
Abbreviation: LDH, lactate dehydrogenase.

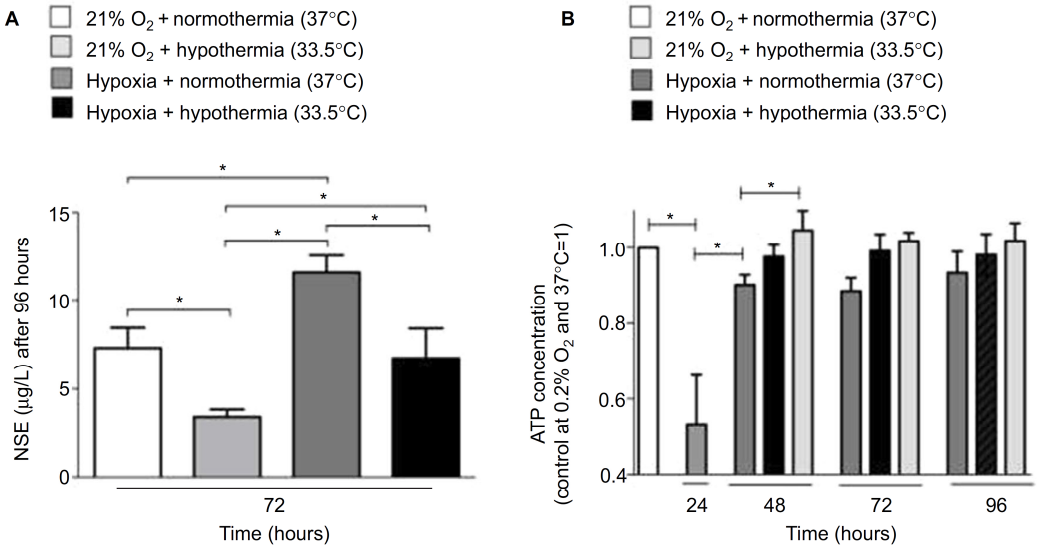

Figure 3 (**A**) NSE release in SK-N-SH cells exposed to hypoxia (0.2% O_2 for 24 hours) followed by normothermia/hypothermia for 72 hours compared to SK-N-SH at atmospheric normoxia and hypothermia/normothermia for 72 hours (n=3, *p<0.05). (**B**) Intracellular ATP concentration in SK-N-SH cells exposed to hypoxia (0.2% O_2 for 24 hours) followed by normothermia/hypothermia compared to SK-N-SH at atmospheric normoxia and hypothermia/normothermia (n=4, *p<0.05).
Abbreviation: NSE, neuron-specific enolase.

ATP

Intracellular ATP concentration was quantified in SK-N-SH cells exposed to hypoxia and then treated with moderate hypothermia (Figure 3B). Hypoxia (0.2% O_2 for 24 hours) significantly decreased ATP levels. Twenty-four hours after reoxygenation, ATP concentration significantly increased and was almost as high as in the control at 21% O_2. Moderate hypothermia for 24, 48, or 72 hours led to slightly increased intracellular ATP levels in SK-N-SH cells already exposed to hypoxia, but the differences were not significant. Neurons that were exposed to hypothermia at atmospheric normoxia (21% O_2) had similar ATP levels as those maintained at 37°C.

Cell morphology is maintained under hypothermic conditions

In addition to decreased viable cell counts, exposure to hypoxia (0.2% O_2 for 24 hours) followed by 72 hours at atmospheric normoxia also led to morphological changes, as observed in swollen and enlarged somata in the SK-N-SH neurons (Figure 4). Treatment with moderate hypothermia (33.5°C) for 72 hours attenuated the morphological changes induced by hypoxia. The cooled cells returned to their physiological appearance, and no sign of cell swelling was observed.

Subatmospheric oxygen tension (8% O_2 for 24 hours followed by 72 hours normoxia) resulted in only small morphological changes (Figure 4). SK-N-SH neuron cell somata were only slightly enlarged, but physiological shape was preserved, and no signs of cell swelling were observed. No observable differences were seen in the morphological appearance of SK-N-SH cells that were cooled after exposure to subatmospheric oxygen tension as compared to those maintained at atmospheric normoxia (21% O_2).

RBM3 mRNA expression (reverse transcriptase-quantitative polymerase chain reaction [RT-qPCR])

Moderate hypothermia for 48 hours resulted in significantly increased levels of RBM3 transcripts (mRNA) in human SK-N-SH cells (Figure 5A). The level of RBM3 mRNA was threefold higher compared to SK-N-SH cells at normothermia. No impact on the regulation of RBM3 transcription by hypoxia or subatmospheric oxygen tension (0.2% or 8% O_2 for 24 hours) was observed (Figure 5B). Moderate hypothermia increased RBM3 mRNA levels in SK-N-SH cells that had undergone hypoxia (0.2% O_2 for 24 hours) beforehand, with significant differences 72 and 96 hours after the experimental start (Figure 6). Likewise, moderate hypothermia resulted in a significant upregulation of RBM3 mRNA in SK-N-SH cells exposed to 8% O_2 for 24 hours before reaching a significant level 48 and 72 hours after the experimental start (Figure 6). The temperature-regulated induction of RBM3 mRNA in post-hypoxic SK-N-SH cells was similar to that in SK-N-SH cells exposed to atmospheric conditions, at all times. Therefore, no additional or attenuating effect of hypoxia or subatmospheric oxygen tension on the regulation due to moderate hypothermia on RBM3 transcription was observed.

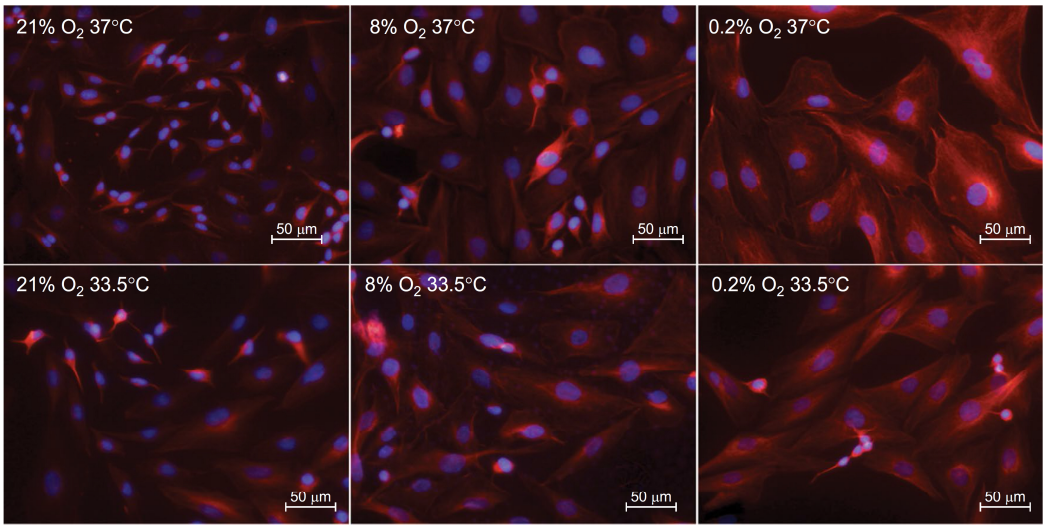

Figure 4 Cell morphology after 24 hours of atmospheric normoxia, subatmospheric oxygen tension, or hypoxia (21%, 8%, or 0.2% O_2) followed by 72 hours of normothermia (37°C) or hypothermia (33.5°C). SK-N-SH cells are stained with anti-beta III-Tubulin (red) and DAPI (blue).
Abbreviation: DAPI, 4′,6-diamidin-2-phenylindol.

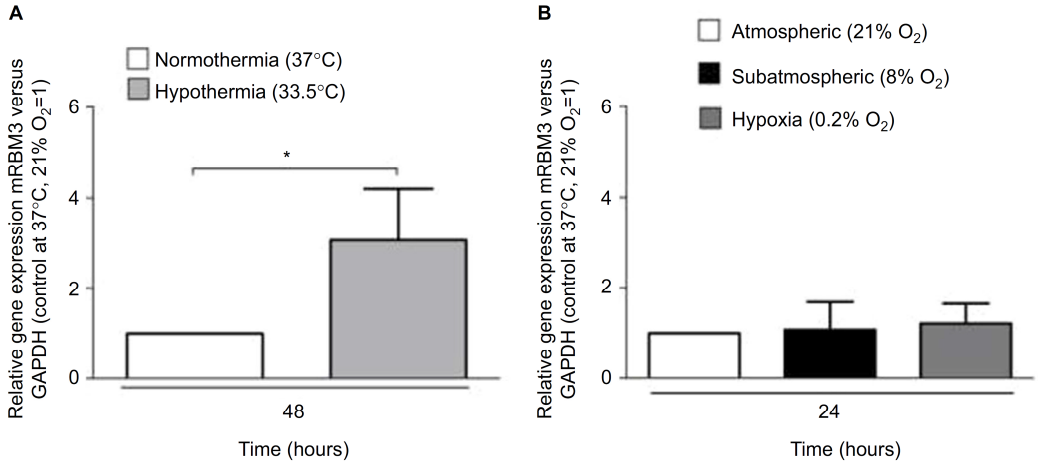

Figure 5 Expression of RBM3 mRNA in SK-N-SH after (**A**) 48 hours of moderate hypothermia compared to a normothermic control group and after (**B**) 24 hours of 0.2% O_2 or 8% O_2 oxygen tension compared to an atmospheric normoxic control group at 21% O_2 (n=3–6, *p<0.05).
Abbreviations: RBM3, RNA-binding motif 3; m, messenger.

RBM3 protein expression (Western blot analysis)

In accordance with the presented RT-qPCR data, RBM3 protein levels were significantly increased in SK-N-SH neurons in response to moderate hypothermia (33.5°C) for 48 hours (Figure 7A). Neither reduction to 0.2% nor to 8% O_2 for 24 hours had an observable impact on RBM3 expression (Figure 7B). Moderate hypothermia (33.5°C) for 24, 48, or 72 hours significantly increased RBM3 protein concentration in SK-N-SH cells that were either exposed to hypoxia or subatmospheric oxygen tension for 24 hours (Figure 8). The overexpression of RBM3 protein in response to moderate hypothermia was similar in SK-N-SH cells already exposed to hypoxia or kept at 21% O_2. No additional effects on RBM3 expression could be observed. The results on RBM3 protein expression correlate with the findings in the regulation of RBM3 transcripts analyzed via RT-qPCR.

Discussion

Cell death

Reperfusion or reoxygenation injury is an acknowledged mechanism of cellular death characterized by excessive formation of ROS, leading to an inflammatory response and consequently cell death.[26] In the present study, the authors demonstrated that reoxygenation after exposure of SK-N-SH

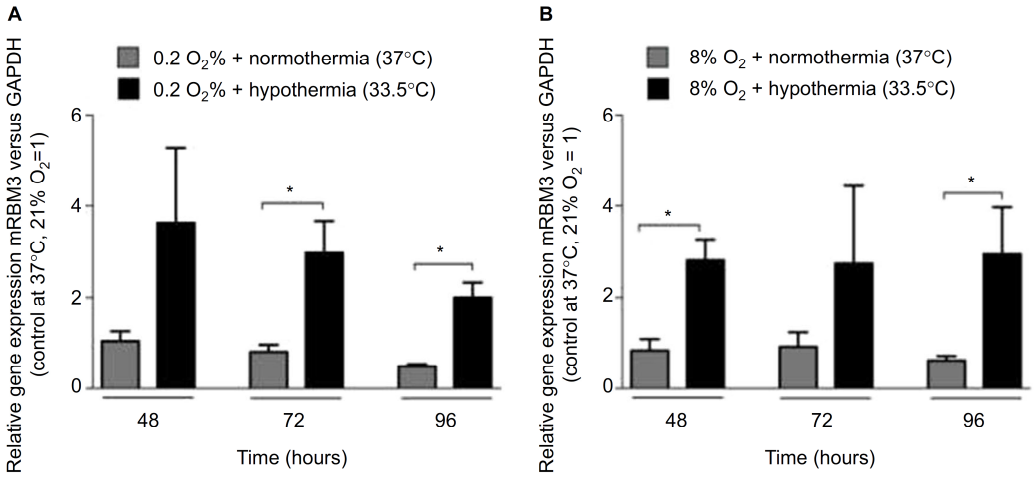

Figure 6 Expression of RBM3 mRNA in SK-N-SH exposed to 0.2% O_2 (**A**) or 8% O_2 (**B**) for 24 hours followed by normothermia/hypothermia for 24, 48, or 72 hours (total 48, 72 and 96 hours, n=3, *p<0.05).
Abbreviation: RBM3, RNA-binding motif 3.

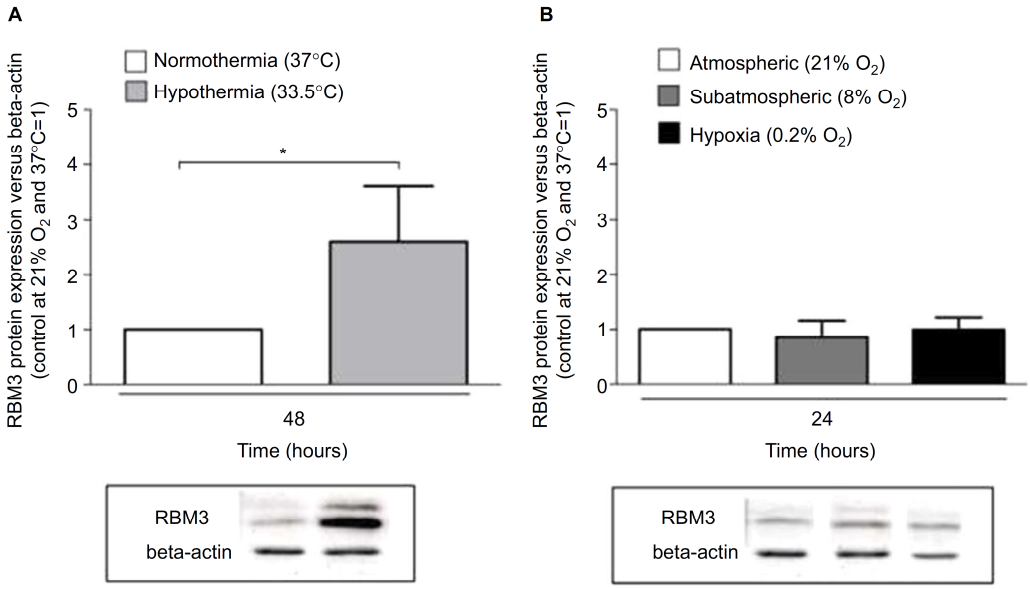

Figure 7 Western blots and densitometric quantification of RBM3 protein versus beta-actin in SK-N-SH after (**A**) 48 hours of normothermia (37°C) versus hypothermia (33.5°C) and after (**B**) 24 hours of 0.2% or 8% O_2 tension compared to 24 hours of atmospheric normoxia (n=4–8, *p<0.05).
Abbreviation: RBM3, RNA-binding motif 3.

neurons to hypoxia (0.2% O_2) for 24 hours results in significantly increased cell death. This effect was not immediately observed at the end of the hypoxic phase, but rather 24 hours after reoxygenation, thus, indicating reoxygenation after hypoxia as the crucial effector of cell death in the authors' cell culture model. Induced cell death was still significantly observable 72 hours after hypoxic insult, as quantified by increased NSE release into the cultured medium. Treatment with moderate hypothermia for 48 and 72 hours effectively attenuated cell death in the post-hypoxic SK-N-SH neurons as observed in significantly reduced LDH and NSE releases, thus further supporting clinical indications of therapeutic hypothermia as a potent neuroprotective strategy against hypoxic–ischemic brain injury. The observations are also supportive of previous in vitro findings where a beneficial effect of mild-to-moderate hypothermia on neuronal cell survival has been demonstrated.[27–29]

Exposure to subatmospheric oxygen tension (8% O_2) for 24 hours, however, did not lead to observable increased cell death in the authors' neuronal cell culture model. The amount of cell death was comparable to neurons maintained at atmospheric normoxia (21% O_2). Although ambient

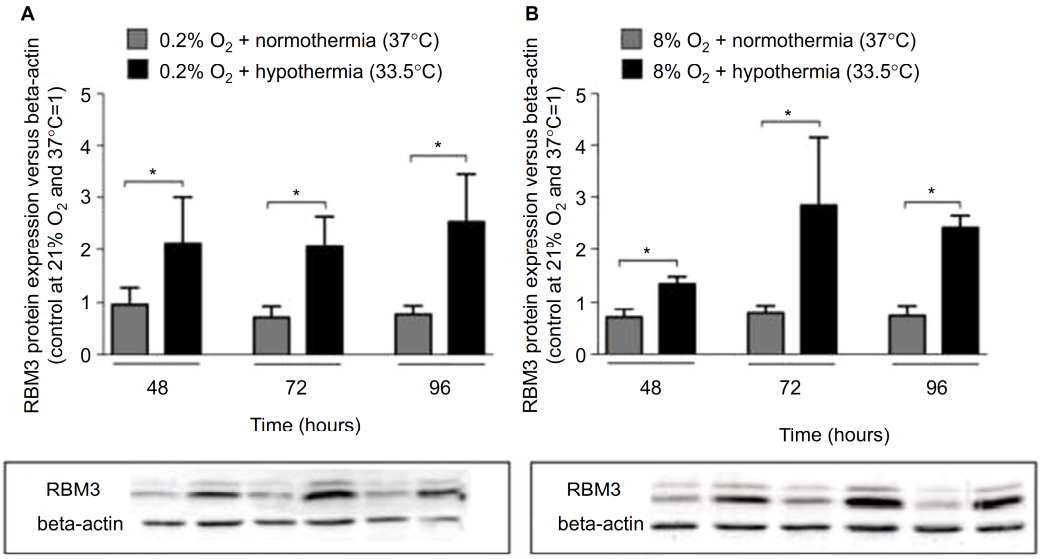

Figure 8 Western blots and densitometric quantification of RBM3 protein versus beta-actin in SK-N-SH cells after (**A**) 24 hours of hypoxia (0.2% O_2) or (**B**) 24 hours of subatmospheric oxygen tension (8% O_2) followed by 24, 48, or 72 hours of normothermia/hypothermia (total 48, 72 and 96 hours, n=4, *$p<0.05$).
Abbreviation: RBM3, RNA-binding motif 3.

oxygen (21% O_2) is commonly used as a normoxic control in the majority of in vitro models, it is higher than in vivo O_2 levels (1%–11%) in the mammalian brain. Previous studies have even demonstrated enhanced survival of neuronal cells under subatmospheric oxygen tension.[30] The findings are comparable to those of Parmar et al who also observed no significant impact of subatmospheric oxygen tension on the number of viable neurons, nor on cell morphology in rat cortices exposed to 8% O_2 for 3 hours.[31] Therefore, it can be concluded that a moderate decrease in oxygen from ambient level has to be considered as a physiological cell environment for neurons and does not result in increased cell death.

Nonetheless, treatment of SK-N-SH neurons exposed to 8% O_2 for 24 hours with moderate hypothermia still resulted in reduced LDH releases and, therefore, attenuated cell death. This cytoprotective effect of hypothermia is also observed in SK-N-SH cells that were only cooled to 33.5°C under atmospheric conditions, and it is suspected that this effect is not due to less cell proliferation in the hypothermic group, as no significant differences in BrdU cell proliferation assay were observed (data not shown). Therefore, it is assumed that hypothermia has a beneficial impact on SK-N-SH cell survival under physiological conditions.

The authors investigated if the observable cell death in the culture model is due to apoptosis via activation of caspase 3 and did not observe any significant differences in the normothermia versus hypothermia group (data not shown). Therefore, cell death in the authors' model is most likely due to necrosis or via a caspase-independent pathway, as has been shown in other studies examining neuronal cell death due to asphyxia.[32]

Intracellular ATP levels are significantly reduced immediately after exposure to hypoxia, but are restored in 24 hours after oxygen deprivation. Even though slightly higher ATP concentrations are observed in post-hypoxic SK-N-SH neurons that were cooled compared to those at 37°C, the difference was not significant, however this cooling may contribute to keeping the neurons viable but does not seem to be the most significant mechanism for neuroprotection induced by moderate hypothermia.

Cell morphology

Cell volume and intracellular ionic concentrations are strictly regulated to maintain a physiological cell function.[33] Due to hypoxia, several changes in cellular pathways occur, leading to ionic imbalances that result in an intracellular accumulation of calcium and sodium. Consequently, normal homeostasis of affected cells is disrupted and fluid streams into the intracellular space result in cell swelling, referred to as osmotic stress.[34] During reoxygenation, this process intensifies the excessive production of ROS. While glial cells are able to regulate their cell volume and ionic balance, neurons are not.[35,36] Thus, osmotic stress leads to neuronal cell death, eventually. These morphological changes could be observed in the authors' cell culture model with human SK-N-SH neurons stained with anti-beta III-Tubulin/DAPI after being exposed to hypoxia (0.2% O_2 for 24 hours) followed by 72 hours of reoxygenation. Moderate hypothermia

(33.5°C for 72 hours) was able to diminish the swelling of cell somata, thus protecting SK-N-SH from hypoxic injury. Subatmospheric oxygen tensions (8% O_2 for 24 hours) did not lead to cell swelling and, therefore, seem to have no impact on ionic balances in human SK-N-SH.

Regulation of RBM3

There is a growing interest in stress responsive genes such as RBM3 which could possibly mediate hypothermia-induced neuroprotection, hence the authors strive to identify key targets of hypothermia to better understand its beneficial effects on cell survival. In the authors' cell culture model, an upregulation of RBM3 transcripts and proteins in response to moderate hypothermia (33.5°C for 24, 48, or 72 hours) was demonstrated. This overexpression was observed in SK-N-SH cells that were exposed to hypoxia or 8% O_2 concentration for 24 hours before induction of hypothermia, as well as in the cells that were kept under atmospheric normoxia at all times. There was no additional effect of hypoxia or 8% O_2 concentration on the upregulation of RBM3 due to moderate hypothermia, and the amount of the increase was similar in all groups (21%, 8%, or 0.2% O_2). The findings are in correlation with previously published data, where the temperature-related regulation of RBM3 was examined. Danno et al[17] observed an increase in RBM3 expression after moderate hypothermia (32°C) in several non-neuronal human cell lines. The authors demonstrated in a previous study an upregulation of RBM3 transcripts and protein in murine hippocampal brain slices and in murine hippocampal neurons in response to moderate hypothermia.[18] This study is, according to the authors' knowledge, the first one to examine the effects of both hypothermia and hypoxia (in different ranges) on the regulation of RBM3 in neuronal cells. Wellmann et al demonstrated a hypoxia-induced upregulation of RBM3 transcripts and proteins in non-neuronal cells in an HIF-1α-independent manner.[19] In contrast, no change in RBM3 expression in the neuronal SK-N-SH cell line in response to hypoxia or subatmospheric oxygen tension (0.2% or 8%) for 24 hours was observed, neither on the transcriptional nor on the translational level. The RBM3 regulation in response to reduced oxygen concentrations in other neuronal cells (murine hippocampal HT-22, murine primary neurons, data not shown) was also examined and the authors could not find any regulation, neither on transcriptional nor on the translational level. Trollmann et al exposed pregnant mice to hypoxia (6% O_2 for 6 hours) at late pregnancy and analyzed the gene expression of the fetal brains and the placenta via microarray analysis.[37] They also did not see an upregulation of RBM3, but saw a suppression of RBM3 expression in response to hypoxia. In conclusion, the oxygen-regulated expression of RBM3 seems to distinguish between different cell types and possibly also depends on other stress factors that occur simultaneously such as additional glucose deprivation during ischemia.

Through which specific molecular mechanism is RBM3 protecting neurons from cell death remains an unanswered question. Prior studies implicate that RBM3 is involved in protein translation, as it is able to bind ribosome subunit 60S and increases the formation of polysomes.[21,22] RBM3 might act as a chaperone to ensure basal protein translation under hypothermic conditions. Moreover, RBM3 seems to have a regulatory function in the expression of microRNAs, although its exact function remains unclear.[38–40] It has been demonstrated that RBM3 is able to mitigate cell death due to neurodegenerative diseases.[25,41] Interestingly, in that context, a recent study identified RTN3, a protein that is involved in synapse function, as a target of RBM3.[42] Taken together, further research is needed to address the possible targets and molecular functions of RBM3.

Conclusion

The presented cell culture model, by using human SK-N-SH neurons, reflects several important clinical aspects of therapeutic hypothermia and is suitable for investigating the induced pathways and gene expression kinetics. The beneficial effects of moderate hypothermia after hypoxic brain injury with regard to cell survival and morphology were demonstrated, which support the findings of previous studies. Moreover, a temperature-related regulation of the cold-shock protein RBM3 correlating with the protective effects of hypothermia was shown. The findings suggest that RBM3 is a promising key target of hypothermia and, therefore, a possible mediator of hypothermia-induced neuroprotection. Further research is needed to elucidate cellular functions and molecular pathways of RBM3 and its role as a cytoprotective effector.

Acknowledgment

Constanze Pfitzer is a participant in the BIH Charité Junior Clinician Scientist Program funded by the Charité – Universitaetsmedizin Berlin and the Berlin Institute of Health. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Disclosure

The authors report no conflicts of interest in this work.

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4.3 Publikation 3 (Krech et al. 2017)

Krech J, Tong G, **Wowro S,** Walker C, Rosenthal LM, Berger F, Schmitt KRL.

„Moderate therapeutic hypothermia induces multimodal protective effects in oxygen-glucose deprivation/reperfusion injured cardiomyocytes."

Mitochondrion, 07/ 2017, 35:1-10.

Impact Factor: 3.2

Mitochondrion 35 (2017) 1–10

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Moderate therapeutic hypothermia induces multimodal protective effects in oxygen-glucose deprivation/reperfusion injured cardiomyocytes

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ARTICLE INFO

Keywords:
Therapeutic hypothermia
Reperfusion injury
Mitochondrial dysfunction
Cardioprotection
Targeted temperature management

ABSTRACT

Objective: Therapeutic hypothermia has been shown to attenuate myocardial cell death due to ischemia/reperfusion injury. However, cellular mechanisms of cooling remain to be elucidated. Especially during reperfusion, mitochondrial dysfunction contributes to cell death by releasing apoptosis inductors. The aim of the present study was to investigate the effects of moderate therapeutic hypothermia (33.5 °C) on mitochondrial mediated apoptosis in ischemia/reperfusion-injured cardiomyocytes.

Methods: Ischemic injury was simulated by oxygen-glucose deprivation for 6 h in glucose/serum-free medium at 0.2% O_2 in mouse atrial HL-1 cardiomyocytes. Simulation of reperfusion was achieved by restoration of nutrients in complete supplemented medium and incubation at 21% O_2. Early application of therapeutic hypothermia, cooling during the oxygen-glucose deprivation phase, was initiated after 3 h of oxygen-glucose deprivation and maintained for 24 h. Mitochondrial membrane integrity was assessed by cytochrome *c* and AIF protein releases. Furthermore, mitochondria were stained with MitoTracker Red and intra-cellular cytochrome *c* localization was visualized by immunofluorescence staining. Moreover, anti-apoptotic Bcl-2 and Hsp70 as well as phagophore promoting LC3-II protein expressions were analyzed by Western-blot analysis.

Results: Therapeutic hypothermia initiated during oxygen-glucose deprivation significantly reduced mitochondrial release of cytochrome *c* and AIF in cardiomyocytes during reperfusion. Secondly, anti-apoptotic Bcl-2/Bax ratio and Hsp70 protein expressions were significantly upregulated due to hypothermia, indicating an inhibition of both caspase-dependent and -independent apoptosis. Furthermore, cardiomyocytes treated with therapeutic hypothermia showed increased LC3-II protein levels associated with the mitochondria during the first 3 h of reperfusion, indicating the initiation of phagophores formation and sequestration of presumably damaged mitochondrion.

Conclusion: Early application of therapeutic hypothermia effectively inhibited cardiomyocyte cell death due to oxygen-glucose deprivation/reperfusion-induced injury via multiple pathways. As hypothermia preserved mitochondrial membrane integrity, which resulted in reduced cytochrome *c* and AIF releases, induction of both caspase-dependent and -independent apoptosis was minimized. Secondly, cooling attenuated intrinsic apoptosis via Hsp70 upregulation and increasing anti-apoptotic Bcl-2/Bax ratio. Moreover, therapeutic hypothermia promoted mitochondrial associated LC3-II during the early phase of reperfusion, possibly leading to the sequestration and degradation of damaged mitochondrion to attenuate the activation of cell death.

1. Introduction

Ischemia of the heart is one of the leading causes of death worldwide (Finegold et al., 2013). Although, early reperfusion from acute myocardial ischemia is considered crucial for the patients' outcome, reperfusion itself causes injury to the myocardium, which can account for up to 50% of the infarct size (Ong et al., 2015). Hence, the myocardial reperfusion phase following ischemia represents an interesting target for cardioprotective intervention.

Due to its electrical and mechanical activity, the heart generates > 90% of ATP from oxidative phosphorylation in the mitochondria. Therefore, mitochondria occupy up to 40% of myocardial intracellular

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http://dx.doi.org/10.1016/j.mito.2017.04.001
Received 1 November 2016; Received in revised form 1 March 2017; Accepted 4 April 2017
Available online 08 April 2017

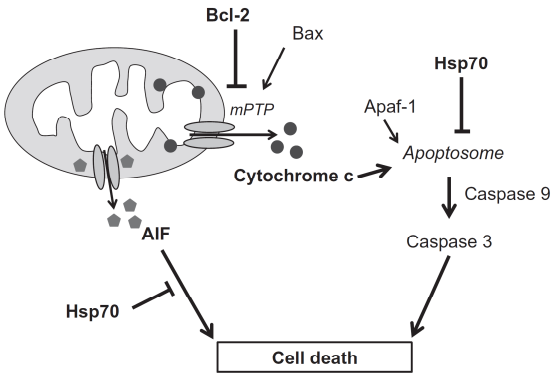

Fig. 1. Synopsis of caspase-dependent and -independent intrinsic apoptosis induction and regulating anti-apoptotic pathways.

volume and the continuous requirement for ATP can only be achieved by aerobic metabolism (Jafri et al., 2001; Kadenbach et al., 2011). Myocardial ischemia/reperfusion (I/R) injury causes intracellular calcium overload, ATP depletion and oxidative stress (Varela et al., 2010; Schafer et al., 2001; Lin et al., 2001). These factors trigger the opening of an unspecific pore in the mitochondrial inner membrane (Uribe-Carvajal et al., 2011). Opening of mitochondrial permeability transition pore (mPTP) mainly occurs during reperfusion as acidic conditions during ischemia antagonizes pore opening (Griffiths and Halestrap, 1995; Di Lisa et al., 2001). Mitochondrial membrane permeabilization through mPTP opening results in loss of mitochondrial membrane potential and ATP depletion. Moreover, opening of the mPTP leads to swelling of mitochondrial matrix and the rupture of the mitochondrial outer membrane (Ong et al., 2015). Thereby, apoptogenic factors such as cytochrome *c* (cyt c) and apoptosis inducing factor (AIF) are released from mitochondrial intermembrane space into the cytosol, resulting in caspase-dependent cell death via caspase 3 activation and caspase-independent cell death, respectively (schematic overview in Fig. 1) (Liu et al., 1996; Susin et al., 1999). Therefore, preserving mitochondrial integrity plays an important role in protection against myocardial I/R injury. Notably, inhibition of mPTP has been an important target for cardioprotection over the last few years.

Autophagy is an important intracellular process for the degradation of undesirable proteins and organelles that is crucial for myocardial vitality. Patients with Danon disease are observed to have a dysregulation of myocardial autophagy leading to hypertrophic cardiomyopathy and malfunctional electrical conduction (Rowland et al., 2016). Notably, this intracellular catabolic metabolism is upregulated during phases of nutrient starvation and hypoxia to ensure mitochondrial ATP production and cell survival (Mizushima et al., 2004; Bellot et al., 2009). However, the role of autophagy in myocardial I/R injury remains controversial. Although studies have shown that autophagy can promote both cell survival and cell death, the autophagic pathway may be an interesting therapeutic target for cardioprotection (Gustafsson and Gottlieb, 2008). As permeabilization of the mitochondrial membrane occurs upstream of both capase-dependent and -independent apoptosis, it is plausible to assume that mitochondrial dysfunction can induce mitophagy or mitochondrial autophagy to not only remove the damaged mitochondria but also to regulate mitochondrial number to comply with current metabolic demands.

Therapeutic hypothermia (TH) has been established to convey neuroprotection in patients suffering from cardiac arrest or neonatal hypoxic-ischemic encephalopathy (N. Engl. J. Med., 2002; Bernard et al., 2002; Shankaran et al., 2005; Azzopardi et al., 2014; Azzopardi et al., 2009). Although, most studies focus on the neuroprotective effects, TH has also been reported to convey cytoprotection against I/R injury in heart and kidney (Pacini et al., 2014; Gotberg et al., 2008; Tissier et al., 2014). Several animal studies reported that TH attenuates myocardial I/R injury as observed in reduced infarct size or improved cardiac function (Gotberg et al., 2008; Chien et al., 1994; Dae et al., 2002; Tissier et al., 2009). Chien et al. observed an increase of 10% in infarct size per degree above 35 °C in blood reperfusion temperature (Chien et al., 1994). However, clinical application of TH has not been able to show this distinct correlation in patients with acute myocardial infarction (Dixon et al., 2002; O'Neill, n.d.; Grines, 2004; Gotberg et al., 2010; Erlinge et al., 2014; Nichol et al., 2015). Numerous in vivo studies have reported TH as an effective cardioprotection treatment against I/R injury when initiated during ischemia, while post-ischemic cooling failed to reduce final infarct sizes (Gotberg et al., 2008; Tissier et al., 2009). As possible keys to maximizing the cardioprotective effect of TH appear to be the time to induction as well as the duration of hypothermia, experimental analysis of the protective mechanisms of hypothermia are crucial for establishing time-temperature management guidelines in patients with myocardial infarction.

We previously demonstrated that I/R injury simulated by exposure to oxygen-glucose deprivation (OGD) and subsequent reoxygenation in complete culture medium resulted in increased cell death and reduced ATP production. We observed a cardioprotective effect of intra-OGD therapeutic hypothermia (IOTH) in HL-1 cardiomyocytes, as indicated by a decrease in apoptotic cell death and mitochondrial impairment, and restored cellular metabolic activity (Tong et al., 2015). However, the effect of therapeutic hypothermia on mitochondria mediated apoptosis and mitophagy in an OGD/R-induced cardiomyocyte injury model remains to be elucidated. Therefore, we investigated the efficacy of moderate therapeutic hypothermia (33.5 °C) to attenuate mitochondria mediated apoptosis and induce mitophagy in a murine cardiomyocyte cell model of ischemia/reperfusion injury.

2. Materials and methods

2.1. HL-1 cell culture

HL-1 cells were obtained from William C. Claycomb, Ph.D. (LSU Health Sciences Center, New Orleans, LA, USA). HL-1 cardiomyocytes are derived from the murine atrial AT-1 tumor cell lineage. They are reported to show spontaneous contractions and a phenotype comparable to adult cardiomyocytes (Claycomb et al., 1998). Culture flasks, Petri dishes, and 12-well plates were precoated with 0.2 μg/cm^2 fibronectin in 0.02% gelatine for 1 h at 37 °C. Cardiomyocytes were cultured at 21% O_2 and 5% CO_2 in Claycomb Medium supplemented with 10% FBS (Sigma-Aldrich Chemie GmbH, Munich, Germany), 100 μl/ml penicillin/streptomycin, 2 mM L-glutamine, and 0.1 mM norepinephrine. Cells were passaged after 80% confluency at 1:2 to 1:5 using trypsin/EDTA (0.05/0.02%, respectively).

2.2. Oxygen-glucose deprivation and reperfusion (OGD/R)

Ischemia was simulated in vitro by deprivation of oxygen and glucose for 6 h in glucose/serum free DMEM at 0.2% O_2 and 5% CO_2 in a CO_2 incubator (Binder, Tuttlingen, Germany) (Tong et al., 2015). Control groups were kept at normoxia (21% O_2) in DMEM containing glucose and 10% FBS. After 6 h of OGD, reperfusion was simulated by restoration of nutrients in complete Claycomb medium and 21% O_2 in all groups. All experimental media were supplemented with 100 μg/ml penicillin/streptomycin and 2 mM L-glutamine.

2.3. Moderate therapeutic hypothermia (TH)

Therapeutic hypothermia is classified into mild hypothermia (36–36.9 °C), moderate hypothermia (32–35.9 °C), and deep hypothermia (< 30 °C). It has been shown that systemic side effects increase with the degree of cooling (Polderman, 2009). We previously established a time-temperature protocol for simulated intra-ischemic moderate TH (33.5 °C) for HL-1 cardiomyocytes, based on the guidelines

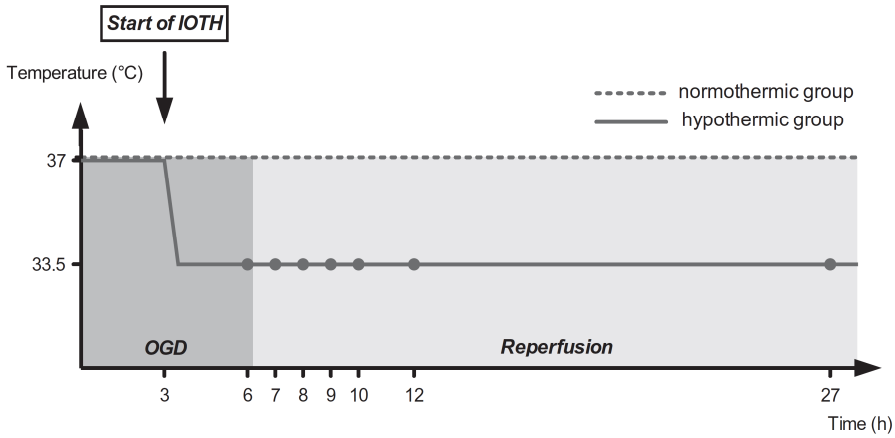

Fig. 2. Time-temperature protocol. Moderate therapeutic hypothermia (33.5 °C) was initiated after 3 h of OGD (IOTH) and cells were maintained at 33.5 °C for up to 27 h. Normothermic experimental groups were maintained at 37 °C for the duration of experiment. HL-1 cells were analyzed directly after OGD at 6 h and after reperfusion at 7, 8, 9, 10, 12, and 27 h.

from the European Resuscitation Council for cardiac arrest survivors (Fig. 2) (Tong et al., 2015; Nolan et al., 2015). Briefly, incubation temperature was decreased to 33.5 °C after 3 h OGD and maintained during simulated reperfusion. Normothermic groups were maintained at 37 °C throughout the duration of the experiment. Samples were analyzed directly after OGD (6 h), every hour during the early reperfusion phase (7–10 h), and beginning and end of the late reperfusion phase (12 and 27 h, respectively) after experimental start in order to thoroughly determine the OGD/R-induced injury and TH-induced cytoprotection.

2.4. Protein extraction and Western-blot analysis

HL-1 cardiomyocytes were seeded in 60 cm^2 Petri dishes at a density of 1.5×10^6 cells/dish 48 h before experimental start. At each time point cells were washed with cold PBS and harvested using a cell scraper. Cytosolic and mitochondrial protein fractions were isolated using the Mitochondria Isolation Kit for Cultured Cells® (Thermo Scientific, Karlsruhe, Germany) according to manufacturer's instructions. Reagents from the kit were supplemented with a protease inhibitor cocktail (1:100, Roche, Mannheim, Germany). To secure a pure mitochondrial fraction, we added two extra washing steps with 1 ml of PBS to the original protocol. Protein concentrations were analyzed using Pierce BCA-Protein Assay (Thermo Scientific, Karlsruhe, Germany). Cytosolic protein fractions (10 µg) were incubated with Pierce Lane Marker Reducing Sample Buffer (Thermo Scientific, Karlsruhe, Germany) at 95 °C for 5 min and loaded onto a 15% gel. Isolated mitochondrion were lysed in 60 µl of reducing sample buffer, incubated at 95 °C for 5 min, and 30 µl of the samples were loaded onto a 15% gel for SDS-PAGE. Subsequently, gels were transferred onto a PVDF membrane (PALL Life Sciences, Port Washington, NY, USA) overnight at 30 mV using a tank blot procedure (Biorad, Munich, Germany). Primary anti-mouse antibodies against Bcl-2, Bax, Hsp70, LC3-I/II, COX IV (1:1000, Cell Signaling, Boston, MA, USA), and β-Actin (1:15,000, Sigma-Aldrich Chemie GmbH, Munich, Germany) were incubated overnight at 4 °C and HRP-conjugated secondary antibodies (anti-rabbit IgG (1:20,000) and anti-mouse IgG (1:10,000), Dianova, Hamburg, Germany) were incubated for 1–2 h at room temperature. We used Dura Super Signal West (Thermo Scientific, Karlsruhe, Germany) to visualize protein expression and Image Lab (Bio-Rad, Munich, Germany) for densitometry analysis.

2.5. Mitochondrial membrane integrity

Mitochondrial membrane integrity was assessed via Western-blot analysis for cyt c and AIF (Cell Signaling, Boston, MA, USA) releases from the mitochondria into the cytosol. Furthermore, we visualized mitochondrial membrane integrity via cyt c immunofluorescence staining using a protocol from Andersen et al. (Andersen et al., 2009). Mitochondria were labeled with MitoTracker Red CMXRos (Invitrogen, Carlsbad, CA, USA), a fluorescent membrane potential dependent dye, which was diluted in glucose free DMEM at 500 nM. 1.5×10^5 cells were seeded in 12-well plates 48 h before experimental start. Cells were first incubated with MitoTracker Red for 30 min at 37 °C, afterwards fixed in 2% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton-X-100 in TBS (15 min at room temperature) and blocked for 1 h (5% BSA in TBS). Cyt c antibody was diluted in 1% BSA at 1:50 and incubated overnight at 4 °C. Secondary antibody conjugated with Alexa Fluor 488 (BioLegend, San Diego, CA, USA) was used at 1:100 and nuclei were stained with DAPI (Sigma-Aldrich Chemie GmbH, Munich, Germany) at 1:4000. Cover slips were mounted using fluorescent mounting medium (Dako Deutschland GmbH, Hamburg, Germany) and visualized using an Axio Imager M1 microscope (Zeiss, Oberkochen, Germany).

2.6. Statistical analysis

Data was analyzed and illustrated using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Results were obtained from at least 4 independent experiments. Groups were compared using one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons. Unpaired Student's *t*-test with Mann Whitney post-test was used for comparison between two groups. Data are presented as means ± SD. P values < 0.05 were considered significant.

3. Results

3.1. Therapeutic hypothermia preserves mitochondrial membrane integrity

Cyt c release into the cytosol is an inductor of the intracellular caspase-dependent apoptosis pathway (Liu et al., 1996). During both early and late reperfusion time points IOTH significantly attenuated cyt c release in OGD/R injured HL-1 cardiomyocytes (Fig. 3A). Furthermore, we observed a significant decrease in AIF release due to IOTH at 10, 12, and 27 h after experimental start (Fig. 3B). AIF release initiates the induction of caspase-independent intrinsic apoptosis (Susin et al., 1999). Mitochondrial membrane integrity was visualized via immunofluorescence staining for cyt c and mitochondria labeling with MitoTracker Red (Fig. 4). OGD/R damaged cells kept at 37 °C showed a diffused distribution of cyt c, whereas distribution of cyt c in the normoxic-control group and in cells treated with IOTH was punctuated

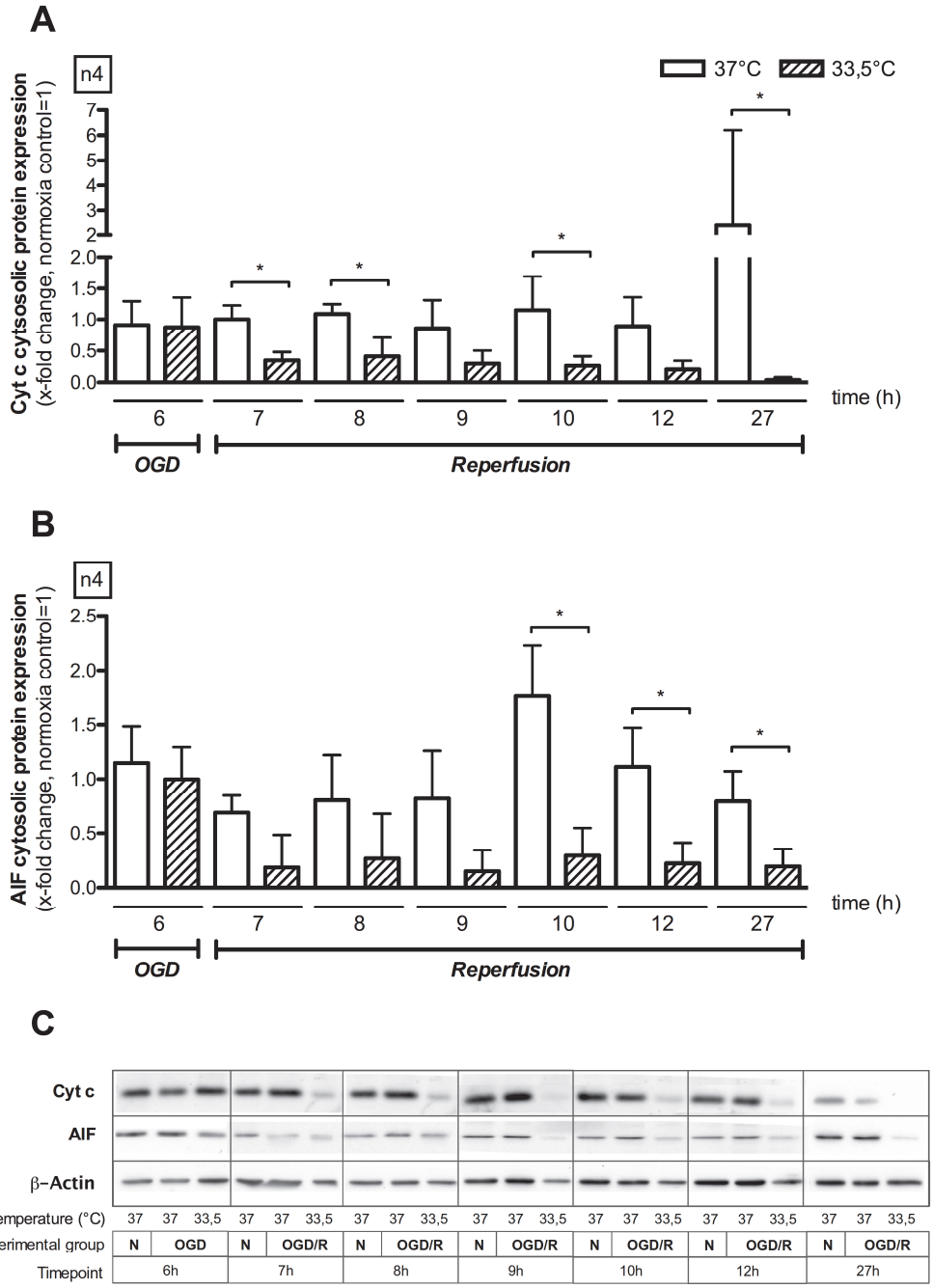

Fig. 3. Effects of intra-OGD therapeutic hypothermia on mitochondrial membrane integrity. Western-blot analysis of (**A**) cyt c and (**B**) AIF releases into the cytosol. Cytoplasmic cyt c and AIF protein expressions are normalized to β-Actin and shown as x-fold changes with normoxic control group at 37 °C = 1. Data from 4 independent experiments is presented as the mean ± SD, $^*p < 0.05$. Panel (**C**) shows representative immunoblots. N = Normoxia, OGD = oxygen-glucose deprivation, R = reperfusion.

and structured. MitoTracker Red accumulates in energized mitochondria due to mitochondrial membrane potential and is an established method for analysis of mitochondria (Indo et al., 2007). Cells maintained at normoxia 37 °C show a structured mitochondrial distribution throughout the cytoplasm of the cardiomyocyte. OGD/R lead to mitochondrial dysfunction and loss of mitochondrial membrane potential, resulting in a quenched staining with MitoTracker Red. However, cells treated with IOTH show a clearly structured mitochondrial morphology occupying most of the cytosol and unquenched staining comparable to the normoxic control group at 37 °C. Our results indicate that the mitochondrial membrane integrity is impaired in OGD/R damaged HL-1 cardiomyocytes. Intra-OGD hypothermia on the other hand, successfully maintained mitochondrial membrane integrity as observed in both Western-blot analysis for cyt c and AIF, as well as immunofluorescence analysis.

3.2. Therapeutic hypothermia promotes anti-apoptotic pathways

Bcl-2 inhibits intrinsic apoptosis, whereas Bax induces this programmed cell death pathway (Nechushtan et al., 1999; Dlugosz et al.,

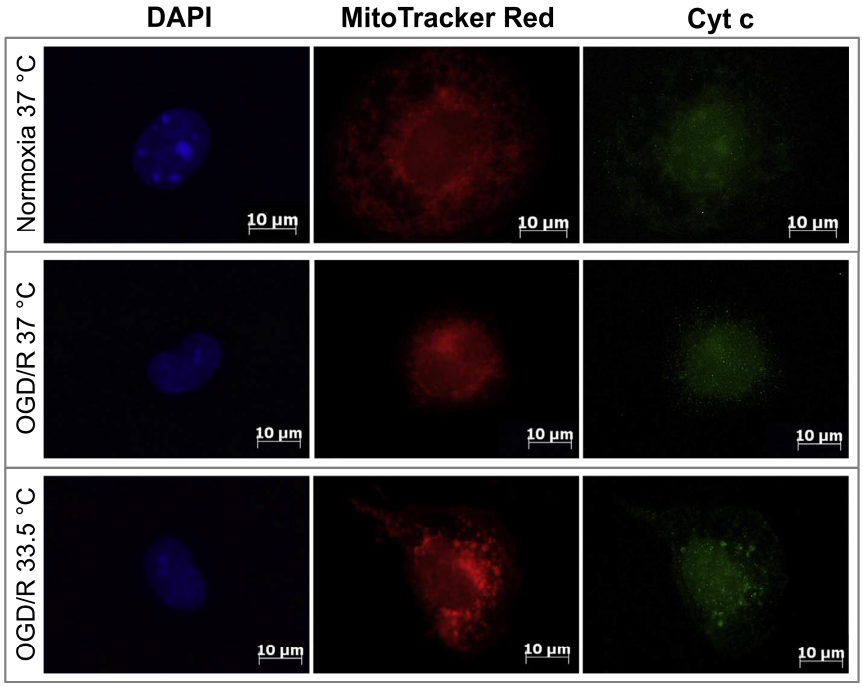

Fig. 4. Immunofluorescence staining as an example of normoxic control group and OGD/R damaged cells following early reperfusion at 37 °C and 33.5 °C. Nuclei are stained with DAPI and mitochondria with MitoTracker Red. Cyt c release into the cytosol is visualized in the last panel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2006). We analyzed the anti-apoptotic ratio of Bcl-2/Bax in mitochondrial protein fraction and observed an upregulation during the reperfusion phase in cardiomyocytes treated with IOTH compared to the corresponding normothermic control with a maximal increase 8 h after experimental start (Fig. 5A). Hsp70 is known to inhibit both caspase-dependent and -independent apoptosis induction (Beere et al., 2000; Choudhury et al., 2011; Ravagnan et al., 2001). Interestingly, we saw a significant increase in Hsp70 expression after 6 h of OGD due to IOTH both in mitochondrial and cytosolic protein fraction (Fig. 5B and C). In summary, intra-OGD therapeutic hypothermia induced two separate anti-apoptotic pathways inhibiting both caspase-dependent and -independent apoptosis in OGD/R injured cardiomyocytes.

3.3. Therapeutic hypothermia promotes mitophagosome formation

LC3-II is involved in autophagosome membrane expansion and fusion and therefore possibly an inductor of the autophagy (Kabeya et al., 2000). Mitophagy or mitochondrial autophagy is marked by clustering of LC3-containing structures onto the surface of damaged mitochondria (Lemasters, 2014). We observed a significant increase in mitochondria associated LC3-II protein in the IOTH treated groups compared to the corresponding normothermic groups during early reperfusion at 7, 8, and 9 h after experimental start that gradually diminished in the later reperfusion phase (Fig. 6).

4. Discussion

Although several studies have shown that therapeutic hypothermia applied during simulated ischemia attenuates cardiomyocyte cell death, the influence of TH on apoptosis induction remains to be elucidated (Tong et al., 2015; Huang et al., 2015; Shao et al., 2010). Apoptosis plays an important role in cardiomyocyte death after ischemia/reperfusion injury (Abbate et al., 2000; Piro et al., 2000). In accordance with other studies, we detected apoptosis induction via caspase 3 activation only during reperfusion (Vanden Hoek et al., 2003; Kawahara et al., 2006). We previously demonstrated that IOTH conveyed cardioprotection against OGD/R-induced injury in HL-1 cardiomyocytes via attenuating caspase 3 activation (Tong et al., 2015). To further explore the impact of IOTH on both caspase-dependent and -independent programmed cell death, we designed the present study analyzing the mitochondrial mediated intrinsic pathway.

We showed that IOTH preserves mitochondrial membrane integrity in HL-1 cardiomyocytes (Fig. 4). These findings indicate that TH attenuates mitochondrial dysfunction comparable to the results of a recent in vitro study showing applied therapeutic hypothermia at 31 °C at the onset of simulated ischemia in H9c2 cardiomyocytes preserves mitochondrial function (Huang et al., 2015). Tissier et al. reported that mild therapeutic hypothermia (32 °C) inhibited calcium-induced formation of mPTP in ischemic rabbit hearts (Tissier et al., 2009). Mitochondrial membrane permeabilization is caused by the opening of mPTP, which results in the release of both cyt c and AIF, thus initiating caspase-dependent and -independent apoptosis. Therefore, preserving mitochondrial membrane integrity is crucial for promoting cell survival after ischemic/reperfusion injury.

In accordance with IOTH preserving mitochondrial membrane integrity, we observed a reduction of cyt c release during early and late reperfusion both by Western-blot and immunofluorescence staining analysis (Figs. 3A and 4, respectively). Cyt c is released into the cytosol due to mitochondrial outer membrane permeabilization (MOMP) and activates Apaf-1, which leads to activation of caspase 9 (Liu et al., 1996; Acehan et al., 2002). Caspase 9 then activates caspase 3 to trigger the intrinsic caspase-dependent programmed cell death pathway (Brentnall et al., 2013). Thus, IOTH significantly reduces cyt c release during reperfusion at 7, 8 and 10 h, which lead to our previous observation of lower activated caspase 3 and less apoptosis induction during the early reperfusion phase (Tong et al., 2015).

Secondly, our latest findings demonstrate that intra-OGD cooling also significantly attenuates caspase-independent apoptosis via the inhibition of AIF, a flavoprotein inserted in the mitochondrial inner membrane, release into the cytosol (Fig. 3B). AIF is released into the

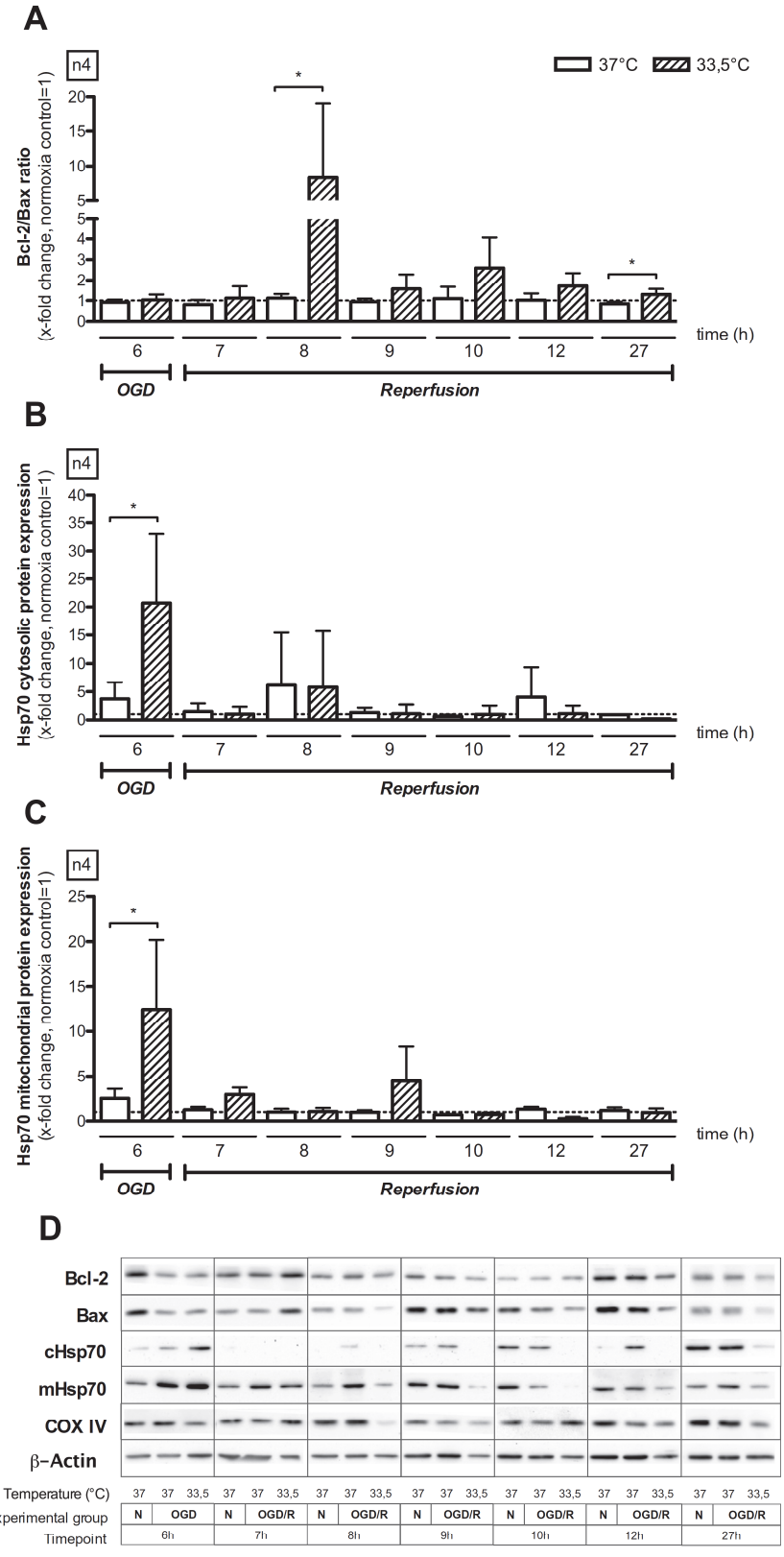

Fig. 5. Effect of intra-OGD therapeutic hypothermia on anti-apoptotic pathways. Western-blot analysis of (**A**) anti-apoptotic Bcl2/Bax ratio. Both (**B**) cytosolic Hsp70 and (**C**) mitochondrial Hsp70 protein expressions were analyzed. Cytosolic protein fraction is normalized against β-Actin, whereas mitochondrial protein expression is normalized against COX IV and shown as x-fold changes with normoxic control group at 37 °C = 1. Data from 4 independent experiments is presented as the mean ± SD, $*p < 0.05$. Panel (**D**) shows representative immunoblots. N = Normoxia, OGD = oxygen-glucose deprivation, R = reperfusion, c = cytosolic, m = mitochondrial.

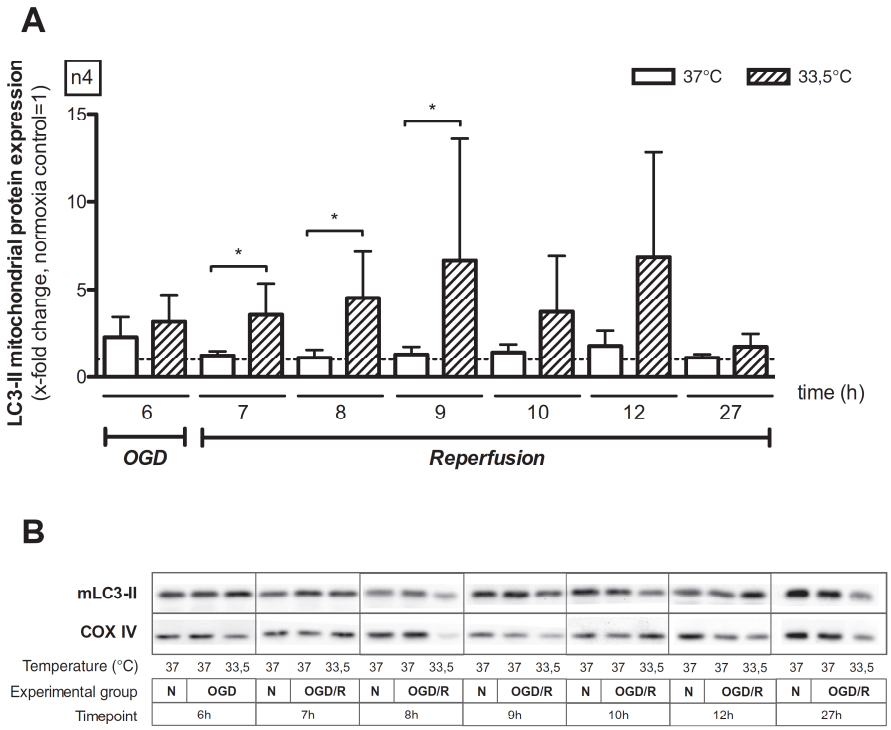

Fig. 6. Effect of intra-OGD therapeutic hypothermia on the autophagic cascade. Western-blot analysis (**A**) of LC3-II is shown as x-fold changes with normoxic control group at 37 °C = 1. Data from 4 independent experiments is presented as the mean ± SD, $^{*}p < 0.05$. Panel (**B**) shows representative immunoblots. N = Normoxia, OGD = oxygen-glucose deprivation, R = reperfusion, m = mitochondrial.

cytosol upon MOMP and translocates to the nucleus to induce chromatin condensation and DNA fragmentation (Daugas et al., 2000). Therapeutic hypothermia attenuates mitochondrial dysfunction induced by simulated reperfusion injury in HL-1 cardiomyocytes and therefore, inhibits intrinsic apoptosis induced by cyt c activation of the caspase cascade and AIF caspase-independent cell death effector. Huang et al. reported a decrease in caspase 9 activation in H9c2 cells by TH, which is comparable to our observed decrease in cyt c release (Huang et al., 2015).

As expected, hypothermia attenuated cyt c release at the beginning of reperfusion, whereas AIF release was significantly decreased during the late reperfusion phase (Fig. 3A and B). It has been shown that AIF release is dependent on the proteolytic cleavage of its mature form (62 kDa) to apoptogenic form (57 kDa), which is released into the cytosol and translocates into the nucleus. Bcl-2 has been shown to attenuate this proteolytic cleavage of AIF, therefore preventing its translocation (Otera et al., 2005). Arnoult et al. observed, that AIF release is dependent on cyt c activation of caspase induced apoptosis (Arnoult et al., 2002). However, it is controversial whether the delayed release of AIF is dependent on caspase-activation and warrants further investigation (Otera et al., 2005; Arnoult et al., 2002).

Moreover, our data strongly suggests that therapeutic hypothermia suppresses apoptosis by the induction of anti-apoptotic proteins such as Bcl-2. The family of Bcl-2 proteins consists of both pro-apoptotic proteins (i.e. Bax, BAD, Bak, and Bok) and anti-apoptotic proteins (i.e. Bcl-2, Bcl-xL, and Bcl-w). The pro-apoptotic Bax plays a crucial role in MOMP. After an apoptotic stimulus Bax translocates from cytosol to the mitochondrial membrane where it oligomerizes and is involved in permeabilizing the mitochondrial outer membrane (Dewson et al., 2012). Bcl-2 is integrated into the mitochondrial outer membrane and inhibits Bax oligomerization, therefore stabilizing the outer mitochondrial membrane (Dlugosz et al., 2006). We observed a significant increase of Bcl-2/Bax ratio in IOTH treated HL-1 cells at early and late reperfusion time points (Fig. 5A). Currently, there is very little data on hypothermia regulated Bcl-2 expression. It has been reported that TH (33 °C) applied after simulated ischemia significantly attenuated protein expression of Bax and increased Bcl-2 expression in HUVECs (Yang et al., 2009). Induced Bcl-2 expression was also observed in an in vitro model of H_2O_2-induced injury in H9c2 cardiomyocytes cooled to 20 °C for 20 min with cardioplegic solution (Drescher et al., 2011). Furthermore, an in vivo study on isolated rabbit hearts showed higher expression levels of Bcl-2 family due to hypothermia (30 °C) (Ning et al., 2002). This suggests that IOTH preserves mitochondrial membrane integrity by Bcl-2 upregulation.

Furthermore, our experimental results show an upregulation of Hsp70 in cytosolic and mitochondrial protein fraction after 6 h of OGD (Fig. 5B and C). It has been shown that Hsp70 conveys protection against protein denaturation due to ischemia, oxidative stress and hyperthermia (Lee et al., 2006; Kabakov et al., 2002; Xu et al., 2009). Several in vivo studies showed that upregulated Hsp70 was able to attenuate I/R-induced cell damage in cardiomyocytes (Suzuki et al., 1997; Marber et al., 1995; Suzuki et al., 2000). Hsp70 was reported to block caspase-dependent apoptosis by preventing the formation of the apoptosome (Beere et al., 2000). Moreover, Hsp70 interferes with AIF translocation into the nucleus, thus inhibiting caspase-independent apoptosis (Choudhury et al., 2011; Ravagnan et al., 2001). Our results suggest that IOTH not only inhibits apoptosis by preserving mitochondrial membrane integrity and therefore preventing apoptosis induction via cyt c and AIF, but also suppresses caspase-dependent and -independent apoptosis directly by attenuating AIF and cyt c releases into to the cytosol. Therefore, early increase in HSP70 protein expression after 6 h of OGD is another possible effector for the significant reduction of cell death in IOTH treated HL-1 cells previously reported by our group.

Autophagy is not only an important catabolic mechanism that ensures cell survival during stressful conditions such as starvation, but is also known to interact with apoptosis induction (Mizushima

et al., 2004; Bellot et al., 2009; Pattingre et al., 2005). Our data shows a significant increase in mitochondria associated LC3-II, which is involved in phagophore formation and elongation, as well as sequestration of presumably damaged mitochondrion for degradation by mitophagy, in the IOTH-treated HL-1 cells during the first 3 h of reperfusion (Fig. 6A) (Kubli and Gustafsson, 2012). Several studies using animal and cell-culture models reported autophagy as a protective mechanism against I/R injury in the myocardium (Yan et al., 2005; Hamacher-Brady et al., 2006; Matsui et al., 2007), while other in vitro studies have reported enhanced cell survival by inhibiting autophagy (Valentim et al., 2006; Aki et al., 2003). Furthermore, the effects of cooling on autophagic activity are controversial. Cheng et al. observed reduced levels of LC3-II during reperfusion in H9c2 cells treated with pre- and post-ischemic moderate hypothermia (32 °C), which correlated with attenuated cell death (Cheng et al., 2013). An in vitro study from Tang et al. on the other hand, reported that deep hypothermia (18 °C) significantly induced autophagy in PC12 cells as assessed by LC3-II and Beclin-1 protein expression. Moreover, apoptosis induction analyzed by protein expression of cyt c, Bax, caspase 3 and caspase 9 was also significantly reduced due to deep hypothermia (Tang et al., 2016). As we report attenuated cell death in the hypothermic group along with increases in mitochondria associated LC3-II as well as anti-apoptotic Bcl-2 and Hsp70, it is reasonable to suggest that IOTH activates the mitophagic process to remove damaged mitochondrion during the early phase of reperfusion (7–9 h). However, as we did not assess Beclin1 or Parkin expressions, we cannot draw a conclusion on regulation of autophagic or mitophagic activity by hypothermia, respectively. Beclin1 is obligatory for inducing autophagy and is known to interact with anti-apoptotic Bcl-2. Beclin1 induces autophagy when dissociated from Bcl-2, leading to a correlation between autophagy and apoptosis (Pattingre et al., 2005). As we did not investigate protein-protein interactions, inhibition of autophagy by Bcl-2 binding to Beclin1 warrants further investigation. We analyzed mitochondrial related Bcl-2 regulation, whereas the interaction between Bcl-2 and autophagy inhibition presumably occurs in the endoplasmatic reticulum (Chang et al., 2010). Therefore, both observed Bcl-2 and LC3-II upregulation via IOTH in the present study are not contradictory. Further investigation of intra-cellular localization of Bcl-2 and especially its interaction with Beclin1 is mandatory for understanding autophagy induction in OGD/R injured cardiomyocytes.

We report various protective mechanisms induced by intra-ischemic initiation of therapeutic hypothermia, however, there are limitations to our study. Our study is based on an in vitro model using a cardiomyocyte cell line, which does not account for the systemic reaction to myocardial ischemia/reperfusion injury in vivo. Although side effects of moderate therapeutic hypothermia are known to be mild and reversible, we did not analyze potential side effects in our presented model (Erlinge et al., 2014). It has been reported that targeted temperature management at 36 °C had the same neuroprotective effect as cooling to 33 °C in patients with out of hospital cardiac arrest (Nielsen et al., 2013). Lastly, we only investigated the effects of cooling and not the effects of rewarming on the cardiomyocytes, which may have deleterious side effects. Therefore, future studies need to analyze the impact of different rewarming rates in order to establish clinical guidelines for the application of therapeutic hypothermia.

5. Conclusion

Currently, there is no satisfactory cardioprotective treatment for patients suffering from myocardial I/R injury. Therapeutic hypothermia has been shown to convey cardioprotection in experimental settings. In order to successfully translate TH as a cardioprotective treatment against myocardial I/R injury to clinical care, an understanding of the underlying mechanisms in action is important. We report that therapeutic hypothermia applied during oxygen-glucose deprivation preserves mitochondrial integrity in cardiomyocytes, which is an important key to protecting the myocardium against I/R injury. The findings of our study suggest that the cardioprotective effects of TH occur concertedly via downregulation of pro-apoptotic stimuli such as cyt c and AIF and induction of anti-apoptotic pathways such as Hsp70, Bcl-2, and autophagy.

Myocardial ischemia/reperfusion injury in vivo is complex and does not only involve cardiomyocyte damage but also results in inflammatory responses and cytokine release. However, the effects of therapeutic hypothermia are various and it has been shown that TH reduces inflammation as well as apoptosis induction (Lee et al., 2016). Both experimental and clinical studies observed that TH can convey cardioprotection against myocardial infarction. As rapid induction of cooling, localization of myocardial infarction and maintenance of therapeutic hypothermia appear to have a major impact on it's cardioprotective efficacy, therapeutic hypothermia as a clinical intervention for myocardial infarction warrants further investigation.

Disclosure

None.

Acknowledgements

We would like to thank Nora Ebermann and Maria Walther for excellent technical assistance. This study was financially supported by Fördergemeinschaft Deutsche Kinderherzzentren E.V., Bonn, Germany [grant number W-B-011/2014].

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5 Lebenslauf (mit Unterschrift)

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

6 Publikationsliste

6.1 Originalarbeiten

Wowro SJ, Tong G, Krech J, Rolfs N, Berger F, Schmitt KRL.; Combined cyclosporin A and hypothermia treatment inhibits activation of BV-2 microglia but induces an inflammatory response in an ischemia/reperfusion hippocampal slice culture model. Front. Cell. Neurosci.; 06/ 2019. (IF 4.30)

Rosenthal LM, Tong G, **Wowro SJ,** Walker C, Pfitzer C, Bottcher W, Miera O, Berger F, Schmitt KRL.; A Prospective Clinical Trial Measuring the Effects of Cardiopulmonary Bypass Under Mild Hypothermia on the Inflammatory Response and Regulation of Cold-Shock Protein RNA-Binding Motif 3. Ther. Hypothermia. Temp. Manag.; 04/ 2019. (IF 2.04)

Krech J, Tong G**, Wowro SJ,** Walker C, Rosenthal LM, Berger F, Schmitt KRL.; Moderate therapeutic hypothermia induces multimodal protective effects in oxygen-glucose deprivation/reperfusion injured cardiomyocytes. Mitochondrion; 07/ 2017. (IF 3.23)

Rosenthal LM, Tong G, Walker C, **Wowro SJ**, Krech J, Pfitzer C, Justus G, Berger F, Schmitt KRL.; Neuroprotection via RNA-binding protein RBM3 expression is regulated by hypothermia but not by hypoxia in human SK-N-SH neurons. Hypoxia; 05/ 2017. (IF N/A)

Plauth A, Geikowski A, Cichon S, **Wowro SJ,** Liedgens L, Rousseau M, Weidner C, Fuhr L, Kliem M, Jenkins G, Lotito S, Wainwright LJ, Sauer S.; Hormetic shifting of redox environment by pro-oxidative resveratrol protects cells against stress. Free Radic. Biol. Med.; 08/ 2016. (IF 5.61)

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Weidner C, Rousseau M, Plauth A, **Wowro SJ**, Fischer C, Abdel-Aziz H, Sauer S.; Iberis amara Extract Induces Intracellular Formation of Reactive Oxygen Species and Inhibits Colon Cancer; PLoS One; 08/ 2016. (IF 2.81)

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Weidner C, **Wowro SJ**, Rousseau M, Freiwald A, Kodelja V, Abdel-Aziz H, Kelber O, Sauer S.; Antidiabetic effects of chamomile flowers extract in obese mice through transcriptional stimulation of nutrient sensors of the peroxisome proliferator-activated receptor (PPAR) family. PLoS One; 11/ 2013. (IF 3.53)

Weidner C, **Wowro SJ,** Freiwald A, Kawamoto K, Witzke A, Kliem M, Siems K, Müller-Kuhrt L, Schroeder FC, Sauer S.; Amorfrutin B is an efficient natural peroxisome proliferator-activated receptor gamma (PPARγ) agonist with potent glucose-lowering properties. Diabetologia; 08/ 2013. (IF 6.88)

Dane AP *, **Wowro SJ ***, Cunningham SC, Alexander IE; Comparison of gene transfer to the murine liver following intraperitoneal and intraportal delivery of hepatotropic AAV pseudo-serotypes. Gene Therapy; 08/ 2012. (IF 4.32)

Hegele A, Kamburov A, Grossmann A, Sourlis C, **Wowro SJ**, Weimann M, Will CL, Pena V, Lührmann R, Stelzl U; Dynamic protein-protein interaction wiring of the human spliceosome. Molecular cell; 02/ 2012. (IF 15.28)

6.2 Poster

Wowro SJ, Tong G, Ebermann N, Lam P, Rolfs N, Schmitt KRL; Assessment of potential strategy for neuroinflammation involving moderate hypothermia and cyclosporin A in an ischemia/reperfusion OHSC model. 10th ISN&N; 10/2018.

Schubert S, **Wowro SJ**, Tong G, Berger F, Schmitt K; Combination of mTOR Inhibitor and Calcineurin Inhibitor in EBV-positive and EBV-negative B-lymphocytes. Annual meeting ISHLT; 04/ 2014.

7 Danksagung

An erster Stelle möchte ich mich bei meiner Doktormutter Prof. Dr. med. Katharina Schmitt für die ausgezeichnete Betreuung, uneingeschränkte Unterstützung, ihr Vertrauen und die vielen hilfreichen Diskussionen bedanken. Vielen Dank für Deine stetige Motivation, Inspiration und für die konstruktiven Gespräche, die meine Weiterentwicklung enorm gefördert haben.

Ich danke all meinen Kolleg*innen, die mich in den letzten Jahren im Labor begleitet haben und den Arbeitsalltag zu etwas ganz besonderen gemacht haben. Ein besonderer Danke vor allem an Dr. Jana Krech, Nele Rolfs, Franka Brey, Phuong Lam, Nalina von Garlen und Maria Walther!

Vielen Danke an Dr. Giang Tong für das Vertrauen und die Unterstützung in meinen Projekten. Danke für Deine großartige Unterstützung und die Hilfe beim Korrekturlesen.

Ein besonders großer Dank gilt Nora Künzel, die mich nicht nur technisch im Labor großartig unterstützt hat und mir immer ratsam zur Seite stand, sondern mir auch eine sehr gute Freundin geworden ist. Die Zeit mit Dir im Labor wird mir fehlen.

Vielen Dank an meine ehemalige Kommilitonin und Freundin Dr. Nadine Paeschke, für die zahlreichen Troubleshootings und den Austausch. Danke für Dein immer offenes Ohr und die gegenseitige Ermutigung und Bestärkung.

Zuletzt danke ich meiner Familie, meinen Eltern Gisela und Helmut, meinen Schwestern Sabine und Emilia und meinen Schwagern Jarek und Paul für die Motivation, die immer willkommene Ablenkung, die bedingungslose Unterstützung und den immerwährenden Rückhalt.