CHAPTER TWO: MATERIALS AND METHODS

2.1 Materials

2.1.1 Laboratory equipments

Balance	Mettler Toledo, Giessen, Germany
Bio Doc Analyzer	Biometra, Goettingen, Germany
Centrifuge, 5415D, 5403	Eppendorf AG, Hamburg, Germany
Computerized densitometry	Biometra GmbH, Goettingen, Germany
Kodak 5000 RA film processor	Eastman Kodak, New York, USA
Freezer, -80 °C	Thermo Forma, Marlotta, USA
Accutrend sensor glucometer	Boehringer, Mannheim, Germany
Heparin-coated catheters	MTB-Medizintechnik, Ulm, Germany
Mini Trans-Blot transfer Cell	Bio-RAD, California, USA
Mini-Protean 3 Cell	Bio-RAD, California, USA
Pipettes, adjustable	Eppendorf AG, Hamburg, Germany
Plate sealer	Genetix, Christchurch, Dorset, UK
Power supply	Biometra, Goettingen, Germany
Pressure transducer	Medex Inc, Carlsbad, USA
Respirator	Ugo Basile, Comerio, Italy
Roller peristaltic pump	Abimed, Langenfeld, Germany
Rotor-stator homogenizer	Ultra Turrax, IKA Analysentechnik GmbH, Germany
Sequence Detection system ABI Prism 7700	Perkin Elmer, California, USA
Spectrophotometer DU 640i	Beckman Coulter, Krefeld, Germany
Thermocycler	Eppendorf AG, Hamburg, Germany
Thermomixer	Eppendorf AG, Hamburg, Germany
Tip-catheter (2F) system	Millar, Föhr Medical Instruments, Seeheim, Germany
Vortexer	Beyer GmbH, Düsseldorf, Germany

2.1.2 Chemicals, reagents, antibodies and kits

Chemical and reagents:

Acrylamide-Bis solution (29:1)	Serva, Heidelberg, Germany
Agarose	Gibco, Karlsruhe, Germany
Ammonium persulfate (APS)	Serva, Heidelberg Germany
Bromophenol blue	Sigma, Deisenhofen, Germany
Chloral hydrate	Sigma, St. Louis, USA
Complete protease inhibitor cocktail	Roche, Mannheim, Germany
Ethidium bromide, 1%	Fluka, Taufkirchen, Germany
Ethylenediaminetetraacetic acid (EDTA)	Merck, Darmstadt, Germany
Gelatin	Merck, Darmstadt, Germany
Glycerol	Merck, Darmstadt, Germany
Glycin	Merck, Darmstadt, Germany
H_2O_2	Sigma, St. Louis, USA
Hematoxylin	Merck, Darmstadt, Germany
Isopropanol	Merck, Darmstadt, Germany
L-NAME	Sigma, St. Louis, USA
N,N,N',N' - tetramethylethylene diamine (TEMED)	Bio-Rad Laboratories, USA
Ponceau S red	Sigma, Deisenhofen, Germany
sodium nitroprusside (SNP)	Sigma, St. Louis, USA
Sodium dodecyl sulfate (SDS)	Sigma, Deisenhofen, Germany
Streptozotocin (STZ)	Sigma, St. Louis, USA
Tissue-tek® O.C.T™ compound	Tissue Tek, Sakura Finetek, USA
Tris base	Merck, Darmstadt, Germany
Trizma hydrochloride (Tris·Cl)	Merck, Darmstadt, Germany
Tween 20	Sigma, Deisenhofen, Germany
β-Mercaptoethanol	Sigma, Deisenhofen, Germany

Enzymes and antibodies

β-Tubulin rabbit polyclonal antibody	Santa Cruz, California, USA
Anti-mouse IgG-HRP	DAKO, Carpinteria, USA
Anti-rabbit IgG-HRP	DAKO, Carpinteria, USA
Biotinylated goat-anti-mouse	Dianova, Hamburg, Germany
eNOS monoclonal mouse antibody	BD Biosciences, San Diego, USA
M-MLV reverse transcriptase	Invitrogen, Karlsruhe, Germany
Mouse-anti-rat ICAM-1	Serotec, Oxford, UK
Mouse-anti-rat VCAM-1	HISS Diagnostics, Freiburg, Germany
NF-κB p65 rabbit polyclonal antibody	Santa Cruz, California, USA
Peroxidase-conjugated streptavidin	Vectastatin ABC Elite, Burlingame, USA
phospho–p44/p42 MAPK (Thr^{202}/Tyr^{204}) rabbit polyclonal antibody	Cell signaling, Beverly, USA
RNase (DNase free)	QIAGEN, Hilden, Germany
Rnasin ribonuclease inhibitor	Promega, Mannheim, Germany

Kits and other materials

Avidin/biotin blocking kit	Vector Laboratories, Burlingame, USA
BCA protein assay	Pierce/KMF, St. Augustin, Germany
Blotting paper, 3 mm	Whatman GmbH, Gottingen, Germany
ECL films	Amersham, Buchinghamshire, UK
ECL plus Western blotting detecton system	Amersham, Buchinghamshire, UK
Lipid enzymatic colorimetric kit	Boehringer, Mannheim, Germany
MicroAmp optical 96-well reaction plates	PE Applied Biosystems, Foster City, USA

phiX174 DNA/BsuRI (HaeIII) Marker	MBI Fermentas, Germany
Polypropylene tubes (15 ml and 50 ml)	Greiner Labortechnik GmbH, Frickenhausen, Germany
PVDF membranes	Bio-Rad, Hercules, CA
Rainbow markers	Amersham, Buchinghamshire, UK
RNeasy Kit	Qiagen , California, USA
SimplyBlue SafeStain .	Invitrogen, Carlsbad, USA
TaqMan universal PCR master mix	PE Applied Biosystems, Foster City, USA
TaqMan® Rodent GAPDH Control Reagents	PE Applied Biosystems, Foster City, USA

Media and Solutions

0.5 M Tris·Cl, pH 6.8	6 g Tris base was dissolved in 60 ml sterile water, adjusted to pH 6.8 with 6 N HCl, Brought total volume to 100 ml with sterile water, stored at 4 °C.
1×TAE	40 mM Tris, 20 mM acetic acid, 1mM EDTA
1.5 M Tris·Cl, pH 8.8	27.23 g Tris base was dissolved in 80 ml sterile water, adjusted to pH 8.8 with 6 N HCl. Brought total volumes to 150 ml with sterile water, and stored at 4 °C.
10% APS	100 mg APS was dissolved in 1 ml of sterile water, stored at -20 °C in 100 µl aliquots.
10×PBS, pH 7.4	80 g NaCl, 2 g KH_2PO_4, 11.1 g Na_2HPO_4, 2 g KCl steril water was added to 1 L, pH adjusted to 7.4, autoclaved.
10×SDS-PAGE running buffer, pH 8.3	30.3 g Tris base, 144.0 g glycine, 100 ml 10% SDS, sterile water was added to 1000 ml.
10×TBS, pH 7.5	10 mM Tris-HCl, pH 7.5, 100 mM NaCl

KHS	NaCl 118.4 mM; KCl 4.7 mM; $CaCl_2$ 2.5 mM; KH_2PO_4 1.2 mM; $MgSO_4$ 1.2 mM; $NaHCO_3$ 25.0 mM; glucose 11 mM.
Protein loading buffer	3.55 ml sterile water, 1.25 ml 0.5 M Tris·Cl (pH 6.8), 2.5 ml glycerol, 2.0 ml 10% (w/v) SDS, 0.2 ml 0.5% (w/v) bromophenol blue, stored at room temperature. 50 µl of β-Mercaptoethanol was added to 950 µl sample buffer prior to use.
Resolving Gel (10%)	4.1 ml sterile water, 3.3 ml 30% acrylamide/Bis, 2.5 ml 1.5 M Tris·Cl (pH 8.8), 0.1 ml 10% SDS. 50 µl 10% APS and 5 µl TEMED were added immediately prior to pouring the gel.
Stacking Gel (5%)	5.7 ml sterile water, 1.7 ml 30% acrylamide/Bis, 2.5 ml 0.5 M Tris·Cl (pH 6.8), 0.1 ml 10% SDS. 50 µl 10% APS and 10 µl TEMED were added immediately prior to pouring the gel.

Computer softwares

ABI primer express software	PE Applied Biosystems, Foster city, USA
Computer image analyzer Lucia G Version 3.52b	Nikon Deutschland GmbH, Düsseldorf, Germany
JMP statistical discovery software version 4.02	SAS Institute, Cary, USA
TINA 2.0 software	Raytest GmbH, Straubenhardt, Germany

2.2 Methods

2.2.1 Experimental animals

Experiments were performed in 8-week-old male Sprague-Dawley (SD) rats with initial weights between 300 and 320 g (Charles River, Berlin, Germany). Rats were housed under standard conditions (20°C, 12-h light/dark cycle) and given free access to water and standard food. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Ethical Committee for the Use of Experimental Animals of the Free University of Berlin (Germany). Diabetes mellitus was induced in 24 animals by a single intraperitoneal injection of STZ (70 mg/kg body weight) dissolved in 100 nM citric acid-trisodium citrate buffer with a pH of 4.5 as previously described [98]. Normal control rats were age-matched and were injected with the same volume of citrate buffer. After 7, 14, 21 and 48 days blood glucose concentrations were measured using a glucometer to confirm successful diabetes mellitus induction and to monitor the diabetic state. Diabetes induction was considered to be successful at blood glucose levels higher than 550 mg dl^{-1}.

2.2.2 Atorvastatin treatment

Rats were randomly separated into 3 groups (n=8 each group): one non-diabetic (SD-Co) and two STZ-induced diabetic groups. Starting 5 days after the STZ injection, STZ-induced diabetic rats were further randomised to receive vehicle (10% β-cyclodextrane solution) (SD-STZ) or were given atorvastatin (50 mg/kg body weight per day by gavage) (STZ-Ator). Treatment was continued for 48 days.

2.2.3 Lipid measurements

At the end of the experimental protocol, blood was withdrawn from the aorta for lipid measurements. The serum triglycerides, total cholesterol, LDL- and HDL- cholesterol levels were measured by an enzymatic colorimetric test on automated clinical chemistry analyzers.

2.2.4 Hindlimb perfusion

For the evaluation of vascular function, the autoperfused hindlimb model (Fig. 1) was taken as described by Angulo *et al*. [99]. Briefly, on treatment day 48, the experimental rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.), and the animals were mechanically ventilated with a respirator and their temperature maintained at 37°C with a thermic table. Under aseptic conditions, Heparin-coated catheters (PP20, internal diameter 0.21 mm) were placed in both carotid arteries. Systemic blood pressure and heart rate were recorded from the left carotid artery through a tip-catheter (2F) system. A femoral artery catheter was placed through a separate incision into the right groin. Perfusion of the right hindlimb was performed by connecting both catheters with a roller

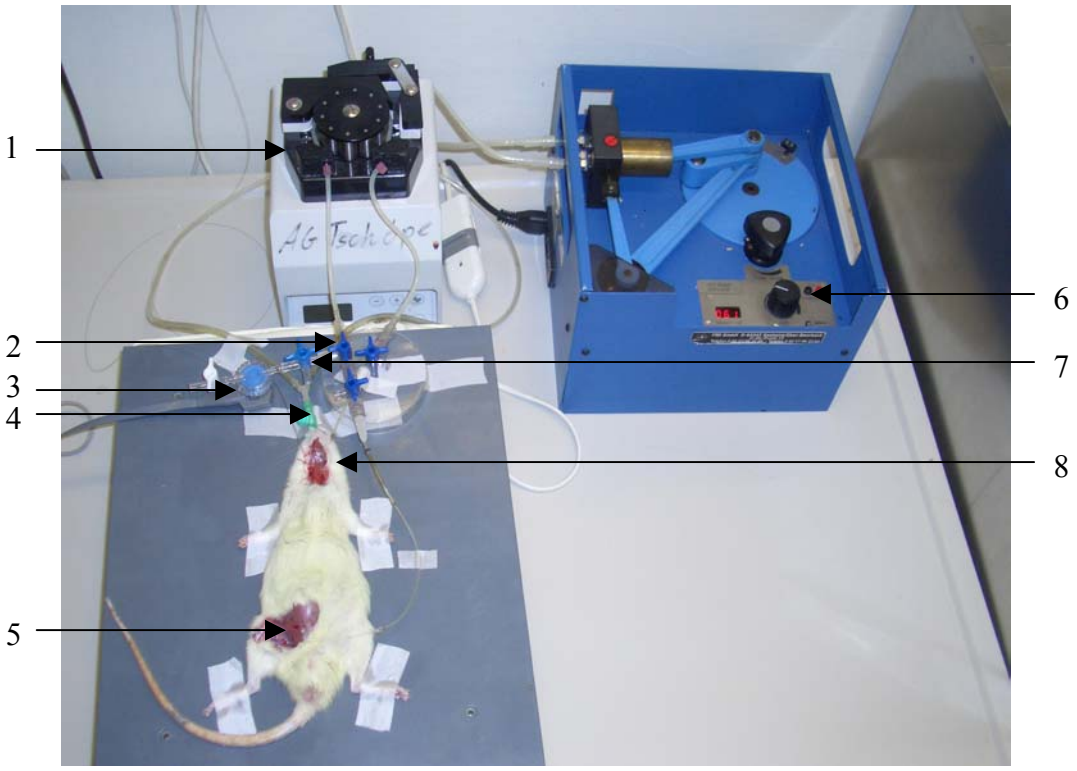

Fig. 1 Model of the autoperfusion hindlimb in an anesthetic rat: 1. Rollerpump; 2. Application pathway; 3. Pressure transducer; 4. Trachea tube; 5. Descendent branch (from the A. femoralis); 6. Respirator; 7. TIP catheter; 8. Ascending branch (from the A. carotis)

peristaltic pump, which delivered blood from the right carotid artery providing a continuous pulsless flow. Flow was set at 2.2 ml/min per kg bodyweight. The flow rate kept constant so that basal vascular resistance to flow could be compared between experimental groups. An infusion pump was connected on the cannula side of the peristaltic pump, thus allowed to obtain depressor responses to infusion of vasoactive drugs: sodium nitroprusside (SNP) or Krebs-Henseleit solution (KHS). The hindlimb perfusion pressure was recorded with a pressure transducer located distal of the peristaltic pump.

2.2.5 Endothelium-dependent and -independent vasodilatation

Infusion of KHS was used to estimate flow-mediated endothelium-dependent vasorelaxation. Extracorporal volume was 1.1 ml, filled with KHS containing sodium heparin (60 IE/ml). The solution consisted of (mM): NaCl 118.4; KCl 4.7; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25.0; glucose 11.1. In order to induce graduated flow-dependent vasodilation, three different volumes of KHS (80, 200 and 600 µl/kg) were administered. In a pilot study we proved that co-treatment with L-NAME (30 mg/kg) inhibited vasodilatation induced by each dose of KHS in healthy animals, indicating the

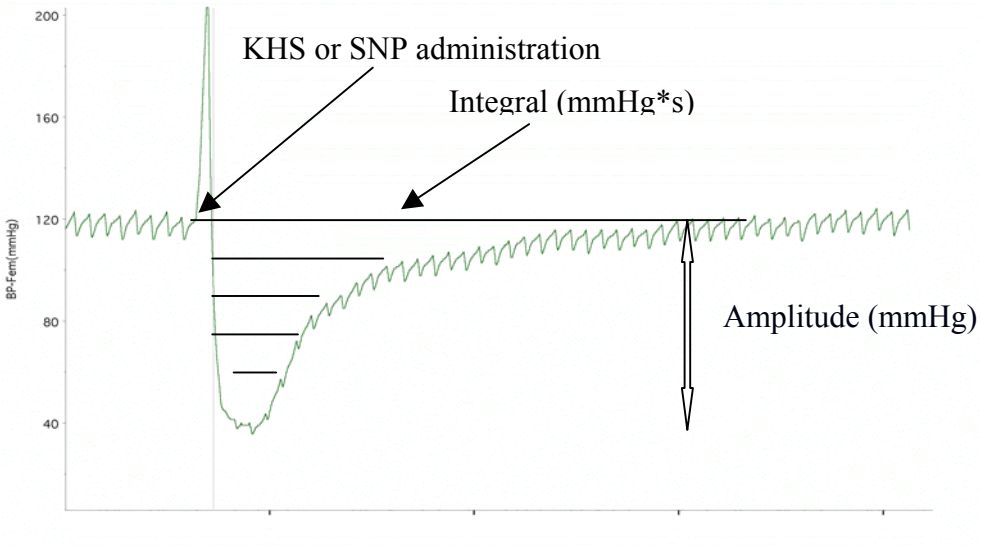

Fig. 2 Digital pressure (in mmHg) against time in axis of the endothelium-dependent vasodilatation

endothelium-dependent mechanism of flow-mediated vascular response in our model. When circulatory parameters again were stabilized, SNP (40 µg/kg) was used to estimate vascular responsiveness to exogenous NO which is endothelium-independent vasodilatation as described by Angulo *et al*, [99]. The rats were anaesthetized throughout the whole measurements. After application of KHS or SNP, the pressure decreased. The vascular response to the stimuli was assessed by calculation the integral (cumulative area, mmHg*s, Fig. 2) of the decreased pressure during the vasodilatation.

2.2.6 Tissue preparation

The rats were sacrificied after the aboved experiment and the non-perfused contralateral quadriceps muscle tissue was isolated, snap-frozen immediately in liquid nitrogen and stored at -80°C.

2.2.7 NAD(P)H oxidase activity

Muscle NAD(P)H oxidase activity was measured by superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction using NADH or NADPH as substrate [100]. The muscles were minced in 10 volumes of ice-cold Tris-sucrose buffer (pH 7.1) containing Tris base 10 mmol/L, sucrose 340 mmol/L, PMSF 1 mmol/L, EDTA 1 mmol/L, leupeptin 10 µg/mL, aprotinin 10 µg/mL, and pepstatin 10 µg/mL. Then the tissue homogenates were sonicated for 20 seconds on ice, followed by extraction for 30 minutes. After centrifugation at 15,000 *g* for 10 minutes, an aliquot (20 µL) of supernatant (50 to 150 µg of protein) was added to the reaction buffer (980 µL) containing cytochrome *c* (78 µmol/L), NADH, or NADPH (100 µmol/L), with or without SOD (1000 U/mL). The samples were then incubated at 37°C for 1 hour, and the absorbance at 550 nm was measured. There was no measurable activity in absence of NADH. The experiments were performed in parallel with and without SOD. A buffer blank was measured in each assay, and SOD-inhibitable cytochrome *c* reduction in buffer blank was subtracted from each sample. NADH-stimulated production of ROS was determined by following increases in cytochrome *c* absorbance. The activity of NAD(P)H oxidase was calculated as SOD-inhibitable cytochrome *c* reduction and expressed as O_2^- in mU mg^{-1} protein.

2.2.8 Real-time reverse transcription-polymerase chain reaction

Gene expression was assessed by TaqMan quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) with the PRISM 7700 Sequence Detection System. Total RNA was isolated from each quadriceps muscle biopsy of rats and one microgram of synthesized complementary DNA (cDNA) was prepared for the measurements.

2.2.8.1 Total RNA extraction

Non-perfused sketel muscles were carefully isolated and cleaned of adhering parenchyma and connective tissue. Total RNA was isolated with a RNeasy Kit from QIAGEN. Briefly, tissues were disrupted and homogenized using rotor-stator homogenizer in buffer RLT with β-ME, the homogenates were further digested with proteinase K solution at 55°C for 10 min. After centrifugation, the supernatants were precipitated with ethanol and the mixture was applied to a RNeasy mini spin column for adsorption of RNA to a membrane. The RNA binded to the membrane after centrifuging for 15 seconds at 10,000 rpm. Contaminants were removed by simple wash spins with buffers RW1 and RPE from kit. After digestion with DNase I to eliminate DNA contamination, the total ready-use RNA was eluted with RNase-free water and was stored at –80°C. The concentration of the sample was determined using a DNA/RNA reader and the quality of RNA was evaluated by A260/A280 value. The integrity and size distribution of total RNA was verified on 1.2% formaldehyde–agarose gel electrophoresis according to the method described in appendix C in Rneasy Mini Handbook.

2.2.8.2 First strand cDNA synthesis

One microgram of total RNA was used to synthesize cDNA in a reaction mixture in a volume of 20 µl which contained 200 units of M-MLV reverse transcriptase in the presence of 1 x first–strand buffer, 5 ng/µl random primers, 10 mM dithiothreitol, 500 µM dNTPs, and 2 units/µl of Rnasin Recombinant Ribonuclease Inhibitor. An additional "no-RT" sample without reverse transcriptase was run in parallel as a control. The reaction was carried out in a 0.5 mL eppendorf tube at 25 °C for 10 minutes, followed by

37 °C for 50 minutes. After reaction, the tube was heated at 70 °C for 15 minutes to inactivate the enzyme and to stop the reaction.

2.2.8.3 Quantitative real-time RT-PCR

The synthesized cDNA was used for PCR amplification. Real-time PCR was carried out by amplification of samples in 96-well plates in an ABI Prism 7700 using specific PCR primer pairs and a dual fluorochrome-labeled hybridization probe (TaqMan probe). The TaqMan probe contains a reporter dye (FAM or VIC) labeled at the 5' end of the probe and a quencher dye (TAMRA) at the 3' end of the probe. During the reaction, the 5'-3' nucleolytic activity of the AmpliTaq Gold enzyme cleaves the probe between the reporter and quencher only if the probe hybrizes to the target. The reporter dye and quencher dye become seperated, resulting in increased fluorescence of the reporter. TaqMan Probe provides a maximal specificity for product identification. During elongation of PCR, more and more dye molecules bind to the newly synthesized DNA. Fluorescence measurement at the end of the elongation step of every PCR cycle was performed to monitor the increasing amount of amplified DNA. The reaction condition was optimized in preliminary experiments using skeletel muscles of SD rats cDNA as a template.

Design of TaqMan probe and primer

For each transcript, specific primers and TaqMan probe were designed using ABI Primer Express software (Table 4) and synthesized by TIB MolBio (Berlin Germany). All primer/probe combinations were designed to span two exons in order to prevent amplification of contaminating genomic DNA. The shortest amplicons (50-150 bps) worked best and gave most consistent results. The following guidelines for TaqMan probe and primer were set after running the software. 1) The G-C content was kept in the 20-80% range; runs of an identical nucleotide, especially for guanine were avoided; 2) For TaqMan probe, G was not put on the 5' end and the melting temperature (Tm) was estimated to be 65-67°C, the strand that contains more Cs than Gs was selected; 3) For primers, Tm should be 58-60°C and the five nucleotides at the 3' end should have no more than two G and/or C bases; 4) The forward and reverse primers were placed as close as possible to the probe without overlapping with the probe. Then a table of possible probes and primers were displayed, first the shortest probe was selected

according above rule and a suitable primer pair was selected to get the shortest amplicon. If possible, a good amplicon was selected to ensure amplification of the target mRNA without co-amplifying the genomic sequence, pseudogenes and related genes. Rodent GAPDH transcripts were amplified in a separate tube to normalize variances in input RNA. The oligonucleotide probes for target mRNA were 5'-joined with the reporter dye 6-FAM and the GAPDH probe was 5'-labeled with VIC.

Table 4 Sequences of Primers/Probes Used for Quantitative Real-time PCR

cDNA		Sequence
eNOS	5'-primer	5'- CCAGGCTGCCTGTGAAACTT -3'
	3'-primer	5'- CTGCGCTTGGGACTGAAGATA -3'
	probe	5'- ATGCCAAGGCTGCTGCCCGAG -3'
ICAM-1	5'-primer	5'- GTCTCATGCCCGTGAAATTATG -3'
	3'-primer	5'- CATTTTCTCCCAGGCATTCTCT -3'
	probe	5'- TCAATCCCTGCATGCCTCCAC -3'
VCAM-1	5'-primer	5'- GGAGGTCTACTCATTCCCTGAAGA -3'
	3'-primer	5'- ACCGTGCAGTTGACAGTGACA -3'
	probe	5'- TGAAATAAGTGGACCACTTGTACACGGGAGA -3'
TNF-α	5'-primer	5'- AGACCCTCACACTCAGATCATCTTC -3'
	3'-primer	5'- CTCCGCTTGGTGGTTTGC -3'
	probe	5'- TCGAGTGACAAGCCCGTAGCCCA -3'
IL-1-β	5'-primer	5'- CTTCGAGATGAACAACAAAAATGC -3'
	3'-primer	5'- GGAGAATACCACTTGTTGGCTTATG -3'
	probe	5'- TGAGCTGAAAGCTCTCCACCTCAATGG -3'

Optimization of real-time PCR condition and quality control

Primers were tested in a standard PCR with 50 ng cDNA as template using the following program: After initial denaturation at 95°C for 4 min, 30 cycle of 95°C 15 sec, 60°C 1 min and 68°C 1 min was performed. Then 8 μl of PCR products were loaded on a 2.5% agarose gel in 1×TAE buffer and run for about 45 min to verify PCR amplification.

In order to get comparable results from experiment vs control, the total RNA samples from different animal quadriceps were freshly isolated in parallel and transferred to

cDNA. In each quantitative real-time RT-PCR, the expressions of both the target gene and the *GAPDH* mRNA, endogenous control were measured in every cDNA samples. Each sample was measured in three replicates. Each PCR run also included nontemplate controls containing all reagents except cDNA to exclude potential contamination. In order to find a suitable concentration of cDNA for real-time PCR, a standard curve was drawn for every batch of cDNA using an aliquot (50 ng of total RNA) of first–strand cDNA diluted serially on the order of twofold. Normally a diluted aliquot (10 ng of total RNA) of the first-strand cDNA was used for the quantification of the target genes.

Quantitative real-time PCR

TaqMan universal PCR master mix reagents provide a PCR mix that is used for designed primers and probe to detect any cDNA sequence. AmpErase uracil-N-glycosylase as a component of the TaqMan master mix can prevent the reamplification of carryover-PCR products by removing any uracil incorporated into double-stranded DNA. Hot-start PCR and time-release PCR were performed to lower background and increase amplification of specific products. The passive reference included in the TaqMan master Mix provides an internal reference to which the reporter-dye signal can be normalized during data analysis. PCR reaction was prepared by mixing 12.5 µl TaqMan universal PCR master Mix (2×), optimized concentration of each of forward and reverse primer, 2.5 µl TaqMan probe (2.5 µM), 5 µl diluted cDNA (10 ng total RNA) and sterile H_2O in total volume 25 µl. The following thermal cycling parameters were used for TaqMan PCR: the reactions were incubated at 50°C for 2 min, and then heated at 95°C 10 min for activation of AmpliTaq Gold, afterwards 40 cycles were performed at 95°C for 15 sec, 60°C for 1 min.

Analysis of quantitative real-time PCR

The fluorescent signal at each cycle generated by the release of fluorophores (FAM) from the quencher (TAMRA) by the 5'-3' exonuclease activity of AmpliTaq polymerase was plotted versus the cycle number. The threshold cycle C(t) value occurs when the sequence detection application begins to detect the increase in signal associated with an exponential growth of PCR product. C(t) was determined for each sample using GeneAmp software. Relative quantification of the target gene was calculated from C(t) values for each well on the real-time PCR plate. Wells with higher initial template

concentrations reach the threshold value at lower cycle numbers during PCR than wells containing lower initial template concentrations. The change in threshold cycle numbers represents difference in initial template concentrations.

The level of target mRNA in various samples was estimated by the relative standard method with a series of dilutions of cDNA from sketel muscls of SD rats. The same preparation of skeletel musle cDNA was used as relative standard in all experiments, thereby allowing comparison of quantitative-PCR data obtained in different runs. Standard curves were generated by linear regression using C(t) versus log concentration.

The cDNA equivalent of experiment samples was calculated from C(t) values using the relative standard curve. All quantifications were normalized to the endogenous control *GAPDH* mRNA to account for variability in the initial concentration. Data were expressed as the ratio between the target gene equivalent and the GAPDH equivalent, yielding the relative expression. No template controls generated C(t) > 40 (i.e. mRNA below the detection level) in all experiments.

2.2.9 Western blot analysis

2.2.9.1 Preparation of protein extract

The non-perfused quadriceps muscle biopsy was homogenised in an ice-cold lysis buffer using a rotor-stator homogenizer. The buffer for homogenisation contained: 1× PBS, 100 μg/mL PMSF, 1% Nondidet P-40, 0.1% SDS, 1mM sodium orhovanadate, and 1 μg/mL aprotinine. After incubation at 4°C for 30 minutes, the homogenate was centrifuged at 10,000 g for 10 minutes at 4°C. The pellet was discarded and the supernatant was collected and frozen at –80°C. The concentration of protein in the supernatant was determined by the BCA protein assay. An equal amount of protein was loaded into a 10% SDS-polyacrylamide gel.

2.2.9.2 SDS-PAGE electrophoresis and protein transfer

Ten percent resolving gel and 5% stacking gel were prepared in a sandwich chamber (7×9 cm). The stacking gel solution was applied on top of the resolving gel and a 1.0 mm comb was inserted into the gel solution. Equal amounts of protein (40 μg) for each sample in 10 μl of supernatant were mixed with 10 μl of 2 X SDS loading buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The protein sample was then denatured by heating at 95°C for 5 minutes and cooled on ice. After removing the comb, 20 μl of denatured protein per lane was separated on a 10% SDS-PAGE. Electrophoresis was carried out at room temperature at 100V for 1 hour. The gel was calibrated with Rainbow markers. After electrophoresis, The gel was equlilbrated for 10 min at room temperature in transfer buffer. A transfer sandwich consisting of the protein gel and a polyvinylidene difluoride (PVDF) membranes was assembled. The proteins were electrophoretically transferred from the gel to the PVDF membranes in the precooled transfer buffer using a Bio-Rad Mini Trans-Blot cell at 4°C, 100V for 1 hour. The efficiency of transfer was confirmed by staining the membrane with ponceau S red. Gel retention was assessed by staining with SimplyBlue SafeStain.

2.2.9.3 Immunoblotting analysis

Nonspecific binding was blocked with 5% nonfat dry milk in Tween-Tris-buffered saline (TBST: 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20). Membranes were probed overnight at 4°C with different antibodies targeting ERK1/2 (p44/p42 MAPK), NF-κB p65 and eNOS. Anti-NF-κB p65 and anti-eNOS were prepared in TBST with 5% nonfat milk, and anti-phospho-p44/p42 MAPK and p44/p42 MAPK were prepared in TBST with 5% BSA. Anti-β-tubulin was used as internal control to verify equal loading of protein. The name and condition of the antibodies used were listed in Table 5. After washed three times with TBST, the probed membrane was then incubated with appropriate HRP-conjugated second antibody for 1 hour at room temperature. Following TBST washing for three times, ECL system was prepared by mixing 40 volumes of the enhanced luminol reagent and one volume of oxidizing reagent for 5 minutes and applied on the membrane (0.125 ml of chelilumineseence reagent per cm^2 of membrane). The

excess chemiluminescence reagent was removed by draining. The membrane was then placed in a plastic sheet protector and exposed to ECL films from 30 seconds to 5 minutes. An optimum exposure was determined according the quality of the developed film. The optical densities of the developed ECL bands of interest were quantitated by computerized densitometry and analyzed with TINA 2.0 software.

Table 5 Antibodies used for immunoblotting analysis

Primary antibody	Dilution	Second antibody	Dilution
Phospho-ERK rabbit polyclonal IgG	1:1000	Goat anti-rabbit HRP-IgG	1:2000
ERK rabbit polyclonal IgG	1:1000	Goat anti-rabbit HRP-IgG	1:2000
eNOS mouse monoclonal IgG	1:2000	Donkey anti mouse HRP-IgG	1:2000
NF-κB p65 rabbit polyclonal IgG	1:500	Goat anti-rabbit HRP-IgG	1:2000
β-Tubulin rabbit polyclonal IgG	1:1000	Goat anti-rabbit HRP-IgG	1:2000

2.2.10 Immunohistochemistry and image analysis

After hemodynamic measurement, tissue samples were removed from the non-perfused contralateral quadriceps muscle, immediately embedded in tissue-tek® O.C.T™ compound, frozen in liquid nitrogen and stored in –80°C. Serial 5-µm-thick cryosections were provided for immunohistochemical analyses. Sections were placed on 10% poly-L-lysine precoated slides. Following fixation in cold acetone, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in PBS. Section were incubated with an avidin/biotin blocking kit. Staining was performed with the following primary antibodies at the given dilutions (45 min, room temperature): mouse-anti-rat ICAM-1 (1:100), mouse-anti-rat VCAM-1 (1:50). After three washes in 1X PBS secondary antibodies were applied for 45 min at room temperature. The first two primary antibodies were detected using a biotinylated goat-anti-mouse IgG (1:400). After washing in 1X PBS as above, sections were incubated with peroxidase-conjugated streptavidin for 30 min at room

temperature. Antigen-antibody complexes were visualized by incubation with substrate solution containing 3-amino-9-ethylcarbazole and peroxidase. Subsequently, the slides were counterstained with Mayer's hematoxylin and mounted in Kaiser's gelatin. The slides were evaluated in a blinded fashion. Using the computer image analyzer Lucia G (Version 3.52b, Nikon), digitalized light micrographs were monitored at 630X magnification and evaluated as described before [101]. First, the net quadriceps muscle area was measured. In this area, positive immunoreactivity area (the red coloured precipitate: chromogen) was marked and measured. The ratio between net quadriceps muscle area and chromogen area was calculated as area fraction (AF).

2.2.11 Statistical analysis

Statistical analysis was performed using JMP statistical discovery software (version 4.02). All data are expressed as the mean ± SEM. One way analysis of variance (ANOVA) in conjunction with Student's *t* test was performed to assess the differences between control, diabetic and atorvastatin treated groups. Differences were considered significant at a *P* value less than or equal to 0.05.