4. DISCUSSION

4.1 EVIDENCE FOR THE OCCURRENCE OF PHGPx IN HUMAN PLATELETS

The present study provides, for the first time, several lines of evidence for the occurrence of PHGPx protein in human platelets. First, Western blotting with two different PHGPx-selective antibodies revealed a band at approximately 23 kDa corresponding to the expected MW of PHGPx. Second, low levels of PHGPx mRNA were detected and third, enzymatic activity with a PHGPx-specific substrate was measured.

PHGPx protein has been found to be widely distributed in the tissue of rats of different ages including the liver, heart, kidney, lung, brain and testis (Zhang *et al*., 1989). Non-selenium glutathione peroxidase activity, however, was present in significant amounts only in the liver, lung and heart of adult animals. PHGPx, GPx-1 and glutathione S-transferase activity have been detected in mouse liver, heart, lung and kidney (Weitzel *et al*., 1990). In the rat pancreatic cell line RINm5F, PHGPx activity was found to be approximately 2× higher than in other cells (Table 1) whereas no GPx-1 activity could be detected. In agreement with the distribution of PHGPx activity, PHGPx mRNA has been detected in the liver, lung, kidney, heart, spleen, brain and testis of rats (Dreher *et al*., 1997; Mizuno *et al*., 2000).

Maiorino *et al*. (1991) first reported on the identification of PHGPx in human cells and found all human tumour cell lines tested contained a phospholipid hydroperoxide peroxidase activity that is accounted for by PHGPx. Similarily, we detected PHGPx activity in all human cell lines tested (Table 1). PHGPx mRNA has been detected in the human cell lines HepG2, CaCo-2 (Wingler *et al*., 1999), A431, human megakaryocytes and UT-7 (this work Fig. 2 and 3). In contrast, Dreher *et al*. (1997) did not detect PHGPx mRNA in the human tissues kidney, heart, liver and lung. Selenoprotein P and GPx-1 transcripts were present in all the human tissues examined. Surprisingly, whereas significant quantities of PHGPx mRNA were detected in human megakaryocytes (Fig.2), no mRNA was detectable in total RNA extracts from human platelets. In a model representing platelet megakaryopoiesis, PHGPx mRNA was found to possess a shorter half-life in differentiated UT-7 cells. Numerous studies (Bermano *et al*., 1995; Lei *et al*., 1995; Sun *et al*., 2001) have demonstrated that PHGPx mRNA levels are insenitive to variations in Se-concentration. mRNAs for selenoproteins require the nonstandard amino acid selenocysteine (Sec; Stadtman, 1996), incorporation of which is dependent on a *cis*-acting mRNA stem-loop structure termed the selenocysteine insertion sequence (SECIS) element as well as a number of *trans*-acting factors. As the Se-

dependent incorporation of Sec at a UGA codon by the SECIS pathway is thought to be in competition with translation termination, it is possible that all selenoprotein mRNAs are natural substrates for the mRNA decay pathway called nonsense-mediated decay (NMD; Maquat, 1995, 2000). The half-life of GPx-1 mRNA has been found to decrease in the liver of animals and cultured cells upon Se-deficiency (Chada *et al*., 1989; Bermano *et al*., 1996; Moriarty *et al*., 1998). However, PHGPx mRNA appears to be insensitive to Se-concentration and NMD (Bermano *et al*., 1995). In NIH3T3 fibroblasts and rat H35 hepatoma cell lines transfected with the pig PHGPx gene, however, PHGPx mRNA is a substrate for NMD and is augmented by Se-deficiency (Sun *et al*., 2001). Endogenous PHGPx mRNA of H35 cells is also subject to NMD. These results may be attributed to a mechanism that either masks or precludes the NMD of PHGPx mRNA in rat liver and testes that is absent in mouse NIH3T3 fibroblasts and rat H35 hepatocytes. An analogues situation may be present in human megakaryocytes and platelets. The mechanism masking/precluding NMD may be lost during the process of megakaryopoiesis. Provided that this model with UT-7 cells represents the *in vivo* conditions, this result affords a plausible explanation for the low levels of PHGPx mRNA in human platelets.

4.2 ROLE OF THE GLUTATHIONE PEROXIDASES IN REGULATING HEPOXILIN SYNTHESIS IN HUMAN PLATELETS

The presence of PHGPx in human platelets implies its possible involvement in the 12-LOX pathway in these cells. Sephadex G-100 fractions containing GPx-1 and PHGPx activity were tested for glutathione-dependent 12-HpETE reductase activity. Both GPx-1 and PHGPx proved to be active (Fig. 11). The ratio of GPx-1:PHGPx activity using 12-HpETE as substrate was found to be approximately 60:1. A similar ratio of activity has been detected in mouse liver (Weitzel *et al*., 1990). These data reveal that among the selenoenzymes GPx-1 is the prominent 12-HpETE reductase. Nevertheless PHGPx may fulfil this function. Although PHGPx contributes to ~ 2% of the total 12-HpETE reductase activity in platelet cytosol, in intact cells it may play a greater role as both 12-LOX and PHGPx activities are membrane bound (Chambers *et al*., 1994, Hagmann *et al*., 1996). This is supported by the work of Ho *et al*. (1997), in GPx-1 knock-out mice no phenotypic changes were observed. In addition, tissues of these mice exhibited neither a lower rate of reduction of exogenously added H_2O_2 nor increased lipid peroxidation in comparison with those of the wild-type mice. Platelets from the GPx-1 knock-out mice did not exhibit an altered AA metabolism in the presence of low AA concentrations (25 μM). In the presence of higher AA concentrations (75 μM) the

platelets from the GPx-1 knock-out mice generated lower levels of 12-HETE with a concomitant increase in polar products in comparison with the wild-type mice platelets. However, no 12-HpETE was detected in either GPx-1 knock-out or wild-type mice suggesting that the contribution of GPx-1 to the cellular antioxidant mechanism under normal animal development and physiological conditions is very limited (Ho *et al*., 1997). Furthermore, Spector *et al*. (2001) found no significant differences in the lens of control and GPx-1 knock-out mice over a period of two years. Organ cultured lenses reduced H_2O_2 at similar rates and the authors concluded that GPx-1 is not required under normal conditions. These results suggest the presence of a second enzyme/system that is capable of fulfilling the role of GPx-1. In contrast, the results of this study with human platelets treated with iodoacetate, which inhibits both GPx-1 and PHGPx, and AA as substrate showed ~ 80% inhibition of 12-HETE formation (Fig. 12, Table 3). Similar qualitative effects have been observed in earlier studies using glutathione-depleting agents (Bryant *et al*., 1982) and in platelets from Se-deficient animals (Bryant *et al*., 1983). This discrepancy between the two sets of results may be explained by the fact that in the absence of GPx-1, PHGPx is predominantly responsible for the reduction of 12-HpETE. These results support the importance of PHGPx as a 12-HpETE reductase. Furthermore, PHGPx has been shown to play a greater role than GPx-1 in regulating the 5-LOX pathway in leukocytes (Weitzel and Wendel, 1993). However, to determine the exact role of PHGPx in tissues and organs, in the reduction of 12-HpETE and as an anti-oxidant, PHGPx/GPx-1 knock-out mice need to be generated.

Although iodoacetate inhibits other enzymes besides GPx-1 and PHGPx, it proved to be appropriate for the investigation of the 12-LOX pathway in platelets as it did not inhibit the 12-LOX activity. This was ascertained by the observation that the sum of the major 12-LOX metabolites analysed were not significantly changed under the conditions used (Table 3). This is in agreement with the work of Chen *et al*. (1997) and Huang *et al*. (1998) who identified PHGPx as the endogenous inhibitor of 12-LOX activity in A431 cells. In the presence of 1 mM iodoacetate, however, 12-LOX activity was recovered resulting in a concomittant 10× increase in 12-HETE levels. These results clearly demonstrate that iodoacetate exhibits no inhibitory effect on 12-LOX activity.

The above results support the assumption that selenoenzymes modulate the arachidonic acid cascade and hydroperoxide tone of platelets. A particular HXA_3 synthase, which selectively

forms HXA_3, has been reported in rat pineal gland, brain hippocampus and skin subcutis layer (Pace-Asciak *et al*., 1993; Reynaud *et al*., 1994a, b). In human neutrophils an enzyme system, which converts 12-HpETE selectively into HXA_3, has been demonstrated (Reynaud and Pace-Asciak, 1997). When human platelets were incubated with AA in the absence of iodoacetate, no hepoxilin could be detected within the limits of this method. However, in the presence of iodoacetate, an accumulation of 12-HpETE and concomitant isomerisation to the hepoxilins was observed (Fig. 15). The pattern of products identified reflected the formation of a mixture of trioxilin A_3 (8,9,12-TriHETrE and 8,11,12-TriHETrE) and hepoxilin B_3 in the ratio 1:3.75. Whereas enzymatic catalysis of 12-HpETE yields mainly HXA_3, non-enzymatic hemin catalysis of 12-HpETE yields both HXA_3 and HXB_3 (Reynaud *et al*., 1994b). Therefore a specific HXA_3 synthase activity in human platelets may be excluded. These results clearly show, however, that 12-HpETE is primarily reduced to 12-HETE by GPx-1 and PHGPx and that the selenocyteine-containing glutathione peroxidases are effective in regulating the 12-LOX pathway and controlling hepoxilin synthesis. This result is not surprising, as both PHGPx and GPx-1 have been shown to regulate the 5-LOX activity. Rats injected with selenium were found to possess an 8-fold higher PHGPx activity as compared to controls and produced significantly less lipoxygenase metabolites than controls (Weitzel and Wendel, 1993). In contrast, stimulation of selenium-deficient rat basophilic leukemia cells resulted in an 8-fold release of lipoxygenase metabolites compared to controls. In rat leukocytes and RBC-2H3 cells PHGPx activity is primarily responsible for the reduction of 5-HpETE and governs the activity of leukocyte 5-LOX via regulating the tone of endogenous hydroperoxides (Weitzel and Wendel, 1993; Imai *et al*., 1998). In monocytic cells, GPx-1 but not PHGPx has been found to regulate the 5-LOX activity (Straif *et al*., 2000) and activity in immature HL-60 cells has been shown to be inhibited by PHGPx (Werz and Steinhilber, 1996). Similarly, it has been reported that PHGPx inhibits 15-LOX activity *in vitro* and that the inhibition is due to the reduction of hydroperoxy lipids that are necessary for the 15-LOX activity (Schnurr *et al*., 1996).

Human platelets incubated with 12-HpETE as substrate, in the absence of iodoacetate, produced a mixture of TXA_3 and HXB_3 (Table 5). Reynaud and Pace-Asciak (1997) demonstrated that fresh human neutrophils selectively formed hepoxilin A_3, with little HXB_3 from 12-HpETE. On the other hand, HXB_3 was found to be the major product when boiled neutrophils were used. These results are indicative of an enzymatic process and the authors propose the presence of an enzyme system in human neutrophils, which converts 12-HpETE

selectively into hepoxilin A_3. From this study it may therefore be concluded that human platelets do not possess an enzyme system responsible for hepoxilin synthesis.

Taken together with the hepoxilin formation results with arachidonic acid as substrate, it may be concluded that hepoxilin formation in human platelets occurs via the non-enzymatic isomerisation of 12-HpETE. The results also suggest that the hepoxilins are only formed in these cells above a certain threshold concentration of 12-HpETE (increased hydroperoxide tone) and that the hepoxilins may function as an oxidative stress induced protective eicosanoid. The lack of detectable hepoxilin formation from AA in untreated cells argues against a role of hepoxilins in these cells under normal physiological conditions.

4.3 DISTINCT ROLES OF THE GLUTATHIONE PEROXIDASES IN REGULATING HEPOXILIN SYNTHESIS IN OTHER CELL TYPES

The megakaryoblast cells UT-7 produced ~ 10× higher concentrations of trioxilin A_3 and hepoxilin B_3 in the presence of iodoacetate and 12-HpETE as compared to the cells treated with 12-HpETE alone (Fig. 20). This result supports the role of the glutathione peroxidases (GPx-1 and PHGPx) in regulating the 12-LOX pathway and hepoxilin synthesis. It has been reported that different mouse organs and cell lines respond individually to selenium depletion and supplementation (Weitzel *et al*., 1990; Brigelius-Flohé *et al*., 1997). Whereas the human T cell clone Jurkat did not respond at all with an increase of GPx-1 or PHGPx activity upon selenium supplementation, GPx-1 activity selectively increased in the murine T cell clone EL4 6.1 upon selenium supplementation. In contrast, rat basophilic leukemia cells incubated under Se-deficiency showed a total loss of GPx-1 activity whereas the PHGPx retained 30% activity (Weitzel and Wendel, 1993). Upon supplementation by selenium, PHGPx activity returned to 100% of the control after 8 h while the GPx-1 activity was restored to 100% after seven days. Whereas no 5-HpETE was detectable in both control and Se-deficient cells after 5 min, significant amounts of 5-HpETE were detected within the first minute in Se-deficient cells. These results show that GPx-1 and PHGPx are differentially regulated by selenium status and that PHGPx is responsible for regulating the lipoxygenase metabolite production rates in these cells. To determine the individual roles of GPx-1 and PHGPx in 12-HpETE reduction *in vivo*, UT-7 cells were grown under conditions of selenium deficiency until no GPx-1 activity was detectable, while PHGPx activity was retained. The cells retained the capacity to reduce 12-HpETE, albeit with a lower enzymatic activity (Fig. 13). These results strongly support the assumption that PHGPx is responsible for the reduction of 12-HpETE in

the absence of GPx-1 and that GPx-1 and PHGPx respond individually to selenium depletion and supplementation in the megakaryoblast cells UT-7.

The role of PHGPx in regulating the 12-LOX pathway in the human epidermoid carcinoma cell line A431 was also examined. It has been reported (Huang *et al*., 1999a) that PHGPx masks the 12-LOX activity in these cells. In agreement with the literature no 12-HETE was detected when AA was used as substrate. This effect is not observed in human platelets that also contain both 12-LOX and PHGPx. The GPx-1 activity is 60× higher than the PHGPx activity in both cell types (Table 1). The only difference between platelets and A431 cells with regards to PHGPx is the intracellular localisation (Arai *et al*., 1999). Surprisingly, no 12-HETE, trioxilin A_3 or HXB_3 was detected in the presence of iodoacetate. These results suggest that the 12-LOX activity in A431 cells is regulated by a non-selenocysteine-containing enzyme, presumably a particulate enzyme (Chen *et al*., 1997). In both untreated and heat inactivated A431 cells trioxilin A_3 and hepoxilin B_3 were detected when 12-HpETE was used as substrate. The heat inactivated cells produced ~ 2× more trioxilin A_3 than the untreated cells. TxA_3 formation in rat skin subcutis cells, brain hippocampal slices and pineal gland is due to a heat-sensitive enzyme and heat-inactivation results in the inhibition of 12-HpETE isomerisation into the hepoxilins (Pace-Asciak *et al*., 1993). The same author (Pace-Asciak *et al*., 1983b) has also shown that lung cytosol possesses the ability to isomerise 12-HpETE into the hepoxilins and that the activity is not destroyed by boiling. The authors proposed that a non-enzymatic (ferri-heme induced) process may be responsible (Pace-Asciak *et al*., 1993). The above results, therefore, suggest a non-enzymatic transformation in A431 cells.

4.4 ROLE OF MAMMALIAN LIPOXYGENASES IN HEPOXILIN FORMATION

As 12-HpETE is the precursor to hepoxilin, the capability of cells to synthesis hepoxilins appears to coincide with the presence of 12-LOX. Hepoxilin formation has been observed in human platelets, *Aplysia* brain and rat lung, liver, pancreas, brain, aorta, pineal gland and subcutis layer. All of these cells/organs have also been shown to possess 12-LOX. Surprisingly, Pace-Asciak and co-workers have reported one exception. It was shown that human neutrophils, which lack 12-LOX, produced large amounts of hepoxilin upon stimulation with calcium ionophore and phorbol ester that induce optimal release of arachidonic acid suggesting the presence of an enzyme system (Dho *et al*., 1990). Similarily, human platelets, which lack 5-LOX, have been shown to convert exogenous leukotriene A_4 to

leukotriene C_4 either by a specific LTC_4 synthase or via a 12-LOX mediated reaction to lipoxins (Edenius *et al*., 1994). A specific 'hepoxilin synthase' (Pace-Asciak *et al*., 1993) as well as various non-enzymatic routes have been reported to catalyse hepoxilin formation. Other enzymes such as 15-LOX (Garssen *et al*., 1971; Bryant *et al*., 1985) and a hydroperoxide isomerase from fungus (Gardner, 1991) have been shown to catalyse the formation of hydroxyepoxy fatty acids. As 12-LOX activity is essential for the formation of the hepoxilins, we investigated the role of the mammalian 12-lipoxygenases in hepoxilin synthesis. To our knowledge this is the first evidence for the possible involvement of 12-LOX in the last step of hepoxilin synthesis. Since 12-LOX exhibits both fatty acid dioxygenase and lipohydroperoxidase activities, a concerted action by both may be expected. Trioxilin A_3 and HXB_3 formation was observed by both platelet-type and porcine leukocyte-type 12-LOX's in the presence of both AA and 12-HpETE (Table 8). An analogues situation has been reported for 5-LOX (Ueda *et al*., 1987, Wiseman *et al*., 1987). Arachidonic acid is oxidised to 5-HpETE and then partitions between reduction to 5-HETE and conversion to leukotriene A_4. Both steps in the formation of LTA_4 are catalysed by the 5-LOX enzyme. It was further shown that 5-HpETE acts as a competitive substrate relative to arachidonic acid, suggesting that the dioxygenase and LTA_4 synthase activity share a common active site. Similarly, purified 15-LOX from rabbit reticulocytes has been shown to possess an epoxyleukotriene activity, converting 15-HpETE to 14, 15-LTA_4 (Bryant *et al*., 1985). It is thus proposed that the 12-LOX is competent for both the dioxygenation of arachidonic acid and the isomerisation of 12-HpETE, in platelets and possibly other mammalian cells, to the hepoxilins by virtue of its genuine and pseudo-hydroperoxidase activities. This role of 12-LOX supports the earlier statement in the literature (Pace-Asciak, 1993) that hepoxilin formation may occur in any cell which possesses 12-LOX.

4.5 REGULATION OF PHGPx mRNA TRANSCRIPTION BY EICOSANOIDS

The afore mentioned observations indicate that hepoxilin formation is regulated at the level of the 12-LOX pathway in a multiple manner. One of the controlling enzymes is PHGPx. The role of the 12-LOX products in regulating PHGPx at the transcription level was therefore examined. Quantitative RT-PCR revealed that the eicosanoids AA, 12-HETE, 12-HpETE, HXA_3 and 5-HpETE caused an increase in PHGPx mRNA levels. The increase in PHGPx mRNA transcription was found to be both concentration (Fig. 22) and time (Fig. 23) dependent. This effect is not cell-specific as seen by the increase in PHGPx mRNA levels in both A431 and HeLa cells, the latter lacking both 12- and 15-LOX. The time course revealed

a biphasic pattern in that PHGPx mRNA levels were upregulated after 4 h and down-regulated after 24 h as compared to the untreated cells. The latter effect is in agreement with the literature (Schnurr *et al*., 1999a, b) where it was shown that the cytokines IL-4 and IL-13 down-regulate PHGPx expression after 24 h in A549 cells. An inverse regulation was observed between 12/15-LOX and PHGPx activity, both *in vitro* and *in vivo*, in that 12/15-LOX was upregulated while PHGPx was down-regulated. As the effect of IL-4 and IL-13 was only determined after 24 h, we investigated the effect of IL-4 on the time profile of PHGPx mRNA levels in A549 cells. A similar biphasic profile was observed. The inverse regulation of PHGPx and lipoxygenases suggests that the enzymes are not only metabolically connected with each other but also with respect to their gene expression. This result is supported by the putative short half-life of the PHGPx mRNA in human platelets, suggesting that the expression of this enzyme is a well-regulated process.

The major 12-LOX pathway product 12-HETE has been reported to play a significant role in the cancer metastasis cascade. The role of the alternative 12-LOX metabolite HXA_3 has not been sufficiently considered, therefore the possible role of HXA_3 in cell proliferation was examined. Hepoxilin A_3 induced cell proliferation, as determined by ^{3}H-thymidine incorporation, albeit to a lower extent than 12-HETE. The cell proliferatory effect of 12-HETE is retarded by upto 24 h when added to cells in DMEM/20% FCS as compared to when added to cells in serum poor medium. This effect may be due to the FCS initially masking or binding the 12-HETE. These results clearly show that HXA_3 plays a role in tumour cell proliferation and the role of HXA_3 in other stages of the cancer metastasis cascade still need to be evaluated.

4.6 BIOLOGICAL ACTIONS OF HXA_3 ON HUMAN NEUTROPHILS

It has been reported for many years that the free acid of HXA_3, unlike its methyl ester, does not enter neutrophils and other cells. This would imply that HXA_3 is only active within the cell that it is formed. Therefore, in the past, most studies on the biological activities of HXA_3 on human neutrophils were conducted with its methyl ester. Human neutrophils do not possess 12-LOX, therefore are unable to synthesis hepoxilin. However, human neutrophils have been shown to possess intracellular binding proteins for hepoxilin. This raised the question, if hepoxilin can not enter human neutrophils why do they possess intracellular binding proteins? Furthermore, since the methyl ester is hydrolysed into the free acid and both forms seem to be active (Reynaud *et al*., 1999), it is difficult to discriminate by what

form of HXA_3 any biological action is exerted; only the effects of the free acid may be considered as biologically relevant.

In the present study ample evidence is provided that HXA_3 is biologically active towards human neutrophils in its unesterified form. It is demonstrated for the first time that HXA_3 is a potent chemotactic agent in the 10 to 100 nM range. This concentration range is comparable with LTB_4 however; the effect was higher for LTB_4. The dose-dependent effect of HXA_3 on chemotaxis revealed a biphasic behaviour in that it was attenuated at higher concentrations. The latter effect, not observed with LTB_4 or fMLP, may be due to an overlap with other effects of HXA_3 on cell signalling in neutrophils. The maximal intensity of the chemotactic effect was, however, markedly higher for LTB_4 than for HXA_3 and fMLP. Under our experimental conditions, HXA_3 is a more potent chemotactic agent than fMLP. The alternative 12-LOX product, 12-HETE, did not exhibit chemotactic activity at all at the concentration range investigated, which is in line with the literature (Goetzl and Pickett, 1980). The G-protein inhibitor pertussis toxin abolished the chemotactic activity. Pertussis toxin has been shown to block the liberation of arachidonic acid and of diacylglycerols by HXA_3 methyl ester (Nigam *et al*., 1990b). Hepoxilin A_3, in contrast to LTB_4 and fMLP, failed to induce aggregation. Within the concentration range 10 nM to 300 nM HXA_3 significantly inhibited fMLP-induced aggregation. Hepoxilin A_3, within the concentration range 1 to 7 μM, caused an immediate transient rise in cytosolic Ca^{2+} by releasing it from intracellular stores, as reported earlier for the methyl ester (Dho *et al*., 1990). The extent of calcium release was lower than that produced by fMLP. A lesser intensity of the eicosanoid-mediated calcium signal has also been observed for lipoxins (Nigam *et al*., 1990a). Therefore, chemotactic activity by HXA_3 appears to be mechanistically different from that exerted by fMLP and LTB_4, moreover, more specific. This is supported by the fact that we failed to detect oxygen uptake and activation of the NADPH oxidase system leading to oxidative burst. The lack of activation of the NADPH oxidase system is in line with the observed absence of activation of protein kinase C. Moreover, the HXA_3-evoked chemotaxis appears to be independent of a cytosolic Ca^{2+} signal, since we failed to observe such a signal at concentrations that exhibit chemotactic activity. It is tempting to speculate that a rise in cytosolic Ca^{2+}, which is observed at higher concentrations of HXA_3, counteracts the chemotactic activity of this eicosanoid. Such an assumption would supply a plausible explanation for the bell-shaped dose-response curve for the chemotactic action. The detection of chemotactic activity towards human neutrophils identifies HXA_3 as a proinflammatory

mediator. Contrary to earlier reports with the methyl ester of HXA_3 (Nigam *et al*., 1990b) where a concomitant liberation of AA was observed, HXA_3 caused only a slight enhancement of the liberation of arachidonic acid. In the same concentration range, however, we also observed a significant attenuation of the fMLP-induced arachidonic acid release by HXA_3, which may be regarded as an anti-inflammatory action. The latter phenomenon could be due to a thapsigargin-like effect of HXA_3 as proposed earlier (Laneuville *et al*., 1993), i.e HXA_3 may block the endoplasmic Ca^{2+}-ATPase pump leading to a depletion of the intracellular Ca^{2+} stores, thus blunting the biological effects of subsequently added fMLP. The lack of induction of arachidonic acid release by HXA_3 may in part be due to the weak intensity of the calcium signal in comparison to fMLP.

Taken together, our data argue against the presence of a membrane barrier for unesterified HXA_3 at both submicromolar and micromolar concentrations of this compound, in opposition to earlier reports (Pace-Asciak, 1994). The present observations also imply that HXA_3, like other eicosanoids, can exert its biological activities elsewhere than in the cell in which it is formed. The putative role of a hepoxilin receptor is supported by the suppression of HXA_3 induced chemotaxis by pertussis toxin. Following on from our work, Reynaud *et al*. (1999), restudied the actions of HXA_3 and its methyl ester and found that the failure to demonstrate biological activity of the free acid was due to an artifact caused by the use of an unsuitable solvent.

5. CONCLUSION

Hepoxilins are formed via the 12-LOX pathway of the AA cascade. This pathway is bifurcated at the level of 12-HpETE into a reduction route yielding 12-HETE and an isomerisation route leading to the hepoxilins. In many cells possessing 12-LOX, 12-HETE formation predominates due to the high glutathione peroxidase (GPx-1 and PHGPx) activity whereas hepoxilin formation is usually very low.

The present study has clearly demonstrated the presence of PHGPx in human platelets, and together with GPx-1, its role in regulating the 12-LOX pathway and hepoxilin synthesis by lowering the hydroperoxide tone. Although PHGPx contributes only approximately 2% of the total 12-HpETE reductase activity, the importance of PHGPx as a 12-HpETE reductase in the absence of GPx-1 has been shown. In platelets, and presumably also in a vareity of other cells possessing 12-LOX, hepoxilin formation may only occur under conditions of selenium deficiency or deficiency of reduced glutathione due to oxidative stress. The resulting effect is an enhanced steady-state level of 12-HpETE and shift in the 12-LOX pathway towards hepoxilin formation. Furthermore, we believe that the GPx-1 is of minor importance for the control of the 12-LOX pathway in platelets. This assumption is supported by first, an earlier study that shows that a monoclonal antibody raised against erythrocyte GPx-1 inhibited the 12-HpETE reductase activity of erythrocyte GPx-1 but not that of platelet cytosol (Chang and Fang, 1990), indicating that a peroxidase other than GPx-1 is competent for the formation of 12-HETE in platelets and second, both 12-LOX and PHGPx activities are membrane bound in intact cells (Chambers *et al*., 1994, Hagmann *et al*., 1996). This is further supported by the experiments with GPx-1 knock-out mice where only minor changes in the arachidonic acid metabolism of platelets was observed (Ho *et al*., 1997), whereas inhibition of both enzymes leads to an 80% inhibition of 12-HETE formation. *Aplysia* neural tissue (Piomelli *et al*., 1989) and rat pineal gland (Reynaud *et al*., 1994b) have been reported to form HXA_3 but not HXB_3 under certain conditions from AA or 12-HpETE. In human platelets however, both hepoxilins were formed and only under conditions of oxidative stress. The pattern of product formation does not support the role of 'Hepoxilin synthase' in human platelets as observed in pineal gland (Pace-Asciak *et al*., 1993, Reynaud *et al*., 1994b). Inasmuch as the putative ’hepoxilin synthase‘ proposed by Pace-Asciak *et al*. (1993) has not been isolated and characterised so far, it remains to be clarified in future studies whether the lipohydroperoxidase activity of a lipoxygenase or a different enzyme is responsible for the isomerisation of 12-HpETE in these tissues.

Phospholipid hydroperoxide glutathione peroxidase has been shown to inhibit 5-LOX activity in B-lymphocytes and immature HL-60 cells (Werz and Steinhilber, 1996) aswell as mask 12-LOX activity in A431 cells (Chen *et al*., 1997; Huang *et al*., 1998). Purified PHGPx has also been shown to inhibit the activity of lipoxygenases and cyclooxygenases (Huang *et al*., 1999a). In the present study the masking effect of PHGPx in A431 cells was not abolished by the selenocytseine-enzyme inhibitor iodoacetate, which inhibits both GPx-1 and PHGPx. These results are in contrast to the unmasking achieved by PHGPx antisense oligonucleotides (Chen *et al*., 2000a). Therefore, the role of PHGPx in masking the 12-LOX activity in A431 cells remains unclear and requires further investigation.

Human neutrophils do not contain a 12-LOX and are thus unable to synthesis HXA_3 from arachidonic acid. A selective *de novo* synthesis of HXA_3 is only possible if external 12-HpETE is supplied (Reynaud and Pace-Asciak, 1997). Neutrophils, however, have been reported to contain an intracellular hepoxilin-binding protein (Reynaud *et al*., 1995a, 1996) that may function as a HXA_3 receptor and mediate the actions observed. This is supported by the loss of biological activity upon treatment of the cells with pertussis toxin. The presence of specific intracellular hepoxilin binding sites (Reynaud *et al*., 1995a,b, 1996) and the present observations imply that HXA_3, like other eicosanoids, can exert its biological activities elsewhere than in the cell in which it is formed. It may be concluded that cell signalling by HXA_3 does not involve PKC-dependent processes, in contrast to that evoked by fMLP. Moreover, the HXA_3-induced chemotaxis appears to be independent of Ca^{2+} as Ca^{2+}-release was not observed at concentrations of HXA_3 that exhibit chemotactic activity. This suggests that HXA_3 acts as a proinflammatory mediator. However, the attenuation of the fMLP-induced AA release and aggregation suggests an anti-inflammatory action. This may be due to HXA_3 blocking the endoplasmic Ca^{2+} ATPase pump leading to a depletion of the intracellular Ca^{2+} stores (Laneuville et al., 1993), so that the biological effects of subsequently added fMLP are blunted.

In numerous diseases, e.g. cardiovascular disease, brain tumuors and multiple sclerosis, oxidative stress has been shown to play a critical role. The concentration range in which the biological effects were observed may be achieved *in vivo* under conditions of oxidative stress or selenium deficiency. The chemotactic activity of HXA_3 may enable these cells to challenge neutrophils to the site of oxidative stress without inducing other cell signals that

may lead to the release of inflammatory mediators. In this way, HXA_3 may function as a stress-induced protective eicosanoid. Taken all together, this data raises new aspects as to the possible biological role of HXA_3, especially in diseases where oxidative stress plays an important role.