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# Metabolism of 5-hydroxy-6,8,11,14-eicosatetraenoic acid by human endothelial cells ☆

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**Abstract**

There is increasing evidence that proinflammatory products of the 5-lipoxygenase pathway play an important role in cardiovascular disease. In the present study, we found that human endothelial cells rapidly oxidize the 5-lipoxygenase product 5*S*-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) to 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), a potent chemoattractant for myeloid cells. 5-Oxo-ETE synthesis is strongly stimulated by oxidative stress. This effect is enhanced following inhibition of the pentose phosphate pathway with dehydroepiandrosterone and is mimicked by diamide, which oxidizes intracellular GSH to GSSG. Conversely, it is blocked by depletion of intracellular GSH/GSSG. The kinetics of $H_2O_2$-induced 5-oxo-ETE synthesis by endothelial cells correlate well with changes in the intracellular levels of GSSG and $NADP^+$. These results suggest that exposure of the endothelium to oxidative stress and inflammation could result in the synthesis of 5-oxo-ETE, which could then induce the infiltration of inflammatory cells into the tissue.


*Keywords:* Eicosanoids; 5-HETE; 5-Oxo-ETE; 5-Hydroxyeicosanoid dehydrogenase; 5-Lipoxygenase; Glutathione; $NADP^+$; Oxidative stress; Inflammation; Pentose phosphate pathway

Recent evidence suggests that products of the 5-lipoxygenase (5-LO) pathway are important mediators in cardiovascular disease [1]. Both 5-LO and 5-LO activating protein (FLAP) are highly expressed in atherosclerotic lesions in humans, principally by inflammatory cells within the lesions [2] with 5-LO expression being higher in symptomatic plaques compared to asymptomatic plaques [3]. Genetic studies have demonstrated a link between a single nucleotide polymorphism in the gene encoding FLAP and the incidence of myocardial infarction and stroke [4] and a FLAP inhibitor was shown to suppress biomarkers associated with increased risk of myocardial infarction [5]. Thus far, research has focused on the production of leukotrienes and their potential role in cardiovascular disease. However, we have shown that another important product is formed by the 5-LO pathway, namely 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) [6]. This substance, which acts through the G protein-coupled OXE receptor [7,8], is a potent chemoattractant for neutrophils [9], eosinophils [10,11], and monocytes [12], and hence could potentially be involved in the infiltration of leukocytes into atherosclerotic lesions as well as into the heart following myocardial infarction. In addition to its effects on cell migration, it elicits a variety of other responses in these cells, including

☆ *Abbreviations:* 5-HEDH, 5-hydroxyeicosanoid dehydrogenase; 5-HETE, 5*S*-hydroxy-6,8,11,14-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; 5-oxo-ETE, 5-oxo-6,8,11,14-eicosatetraenoic acid; 13-HODE, 13*S*-hydroxy-9,11-octadecadienoic acid; BCNU, 1,2-bis[2-chloroethyl]-1-nitrosourea; DHEA, dehydroepiandrosterone; HAEC, human aortic endothelial cells; NEM, *N*-ethylmaleimide; $PGB_2$, prostaglandin $B_2$; PMS, phenazine methosulfate; PPP, pentose phosphate pathway; *t*BuOOH, *tert*-butyl hydroperoxide.

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GM-CSF release from monocytes [13], calcium mobilization and CD11b expression in neutrophils and eosinophils, and actin polymerization in all three cell types [6].

5-Oxo-ETE is formed by the oxidation of the 5-LO product 5*S*-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) by 5-hydroxyeicosanoid dehydrogenase (5-HEDH), which is highly selective for this substrate and requires $NADP^+$ as a cofactor [14]. 5-HEDH activity has been detected in leukocytes and platelets [6], but has not previously been reported in structural cells. Although only small amounts of 5-oxo-ETE are synthesized by resting leukocytes, activation of the respiratory burst in phagocytic cells or exposure of mononuclear cells to oxidative stress dramatically increases its rate of formation [6,15].

Endothelial cells are at the interface between blood and tissues, and are intimately involved in the passage of leukocytes into the tissues. The objective of the present study was to establish whether these cells have the ability to synthesize 5-oxo-ETE and, if so, to determine whether this process is affected by oxidative stress, which often accompanies cardiovascular disease.

## Materials and methods

*Materials.* 5-HETE [16] and 5-oxo-ETE [17] were prepared by total organic synthesis, whereas 13*S*-hydroxy-9,11-octadecadienoic acid (13-HODE) was prepared by incubating linoleic acid (Nuchek Prep Inc., Elysian, MN) with soybean lipoxygenase (Sigma–Aldrich, St. Louis, MO) [18]. Prostaglandin $B_2$ ($PGB_2$) was from Cayman Chemical, Ann Arbor, MI. Other reagents were from Sigma–Aldrich.

*Endothelial cells.* Human aortic endothelial cells (HAEC) were obtained from Cambrex (San Diego, CA) and cultured in endothelial growth medium 2 (EGM-2; Cambrex), containing the recommended growth factors, cytokines, and supplements, along with 2% FBS. Cells were subcultured at a seeding density of 2500–5000 cells/$cm^2$ and passaged (up to passage 7) when they reached ~75% confluence.

*Production of 5-oxo-ETE by endothelial cells.* Endothelial cells were grown to about 80% confluence in 6-well plates. The medium was then removed and replaced with indicator-free HBSS (1 ml/well) prior to incubation with 5-HETE (1 μM). After various times, the medium was removed and ice-cold MeOH (0.65 ml) was added. The medium and MeOH fractions were combined and either $PGB_2$ (100 ng) or 13-HODE (100 ng) was added as an internal standard. Cell numbers were determined in selected wells following trypsinization using a hemocytometer.

*Analysis of 5-oxo-ETE.* 5-Oxo-ETE was measured by automated pre-column extraction coupled to reversed-phase-high performance liquid chromatography (RP-HPLC) as described previously [19].

*Measurement of $NADP^+$.* $NADP^+$ was converted to a fluorescent naphthyridine derivative (cf. Ref. [20]) and measured by RP-HPLC. HAEC cultured in 6-well plates were incubated with $H_2O_2$. After various times, the medium was removed, and an ice-cold solution containing 50 mM acetophenone and 3 N KOH in 50% MeOH was added, along with deamino-$NAD^+$ (100 ng in 10 μl PBS) as an internal standard. After 20 min at 0 °C formic acid (62.5 μl) was added. Five minutes later the mixture was extracted with ethyl acetate (2 × 1.5 ml) and the aqueous phase treated with phenazine methosulfate (PMS) (100 μM). Aliquots (50 μl) were subjected to RP-HPLC on a 15 cm column of Ultracarb ODS (5 μm particle size; Phenomenex, Torrance, CA). The mobile phase (flow rate 1.25 ml/min) was a gradient between solvent C (100 mM citric acid containing 4 mM tetrabutyl ammonium bisulfate) and solvent D (acetonitrile) as follows: 0 min, 1% D; 12 min, 25% D; and 14.3 min, 25% D). The $NADP^+$-acetophenone adduct was measured using a Waters model 2475 fluorescence detector ($\lambda_{ex}$, 371 nm; $\lambda_{em}$, 438 nm). The ratios of peak areas for $NADP^+$ and deamino-$NAD^+$ were calculated and the amount of $NADP^+$ determined from a standard curve.

*Measurement of GSH and GSSG.* GSH and GSSG were measured by RP-HPLC by modification of a post-column derivatization procedure in the literature [21]. Following incubation of HAEC with $H_2O_2$ in 6-well plates, the medium was removed and 200 mM phosphoric acid (1 ml) containing 12 mM CHAPS at 0 °C was added. GSH and GSSG levels were determined in 50 μl aliquots by RP-HPLC using a gradient (flow rate, 1 ml/min) prepared from solvents E (0.05% trifluoroacetic acid in water) and F (0.05% trifluoroacetic acid in acetonitrile) as follows: 0 min, 0% F; 10 min, 15% F. The stationary phase was an Ultracarb column as described above. The column eluate was mixed with *o*-phthalaldehyde (370 μM) in 0.2 M $Na_3PO_4$, pH 12 (flow rate, 1 ml/min), and then passed through a loop of PEEK tubing (6 m × 0.5 mm, i.d.; volume, 1.2 ml) in a water bath at 70 °C. Under these conditions both GSH and GSSG were converted to an isoindole adduct, which was measured using a fluorescence detector ($\lambda_{ex}$, 336 nm; $\lambda_{em}$, 420 nm). The amounts of GSH and GSSG were determined from a standard curve using the authentic compounds as external standards.

*Data analysis.* Values shown are means ± SE. Statistical significance was assessed using either one-way or two-way ANOVA as appropriate with the Tukey test as a multiple comparison procedure. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

## Results

### *Metabolism of 5-HETE by endothelial cells*

To determine whether endothelial cells have the ability to synthesize 5-oxo-ETE we incubated HAEC with 5-HETE (1 μM) in the presence of PMS (100 μM), which oxidizes intracellular NADPH to $NADP^+$ [22]. As shown by the chromatogram in Fig. 1A, HAEC possess a high degree of 5-HEDH activity, converting about one-third of the 5-HETE to 5-oxo-ETE. No other metabolites of 5-HETE were detected, either in the presence or absence (data not shown) of PMS.

### *Effects of oxidative stress on the metabolism of 5-HETE by endothelial cells*

HAEC were incubated with 5-HETE in the presence or absence of $H_2O_2$ for different times. In the absence of $H_2O_2$, endothelial cells synthesized modest amounts of 5-oxo-ETE (Fig. 1B). However, $H_2O_2$ strongly stimulated its formation by four to fivefold compared to vehicle-treated controls at all time points investigated.

The concentration–response curve for $H_2O_2$ is shown in Fig. 1C. $H_2O_2$ is a potent stimulator of 5-oxo-ETE formation with an $EC_{50}$ of about 13 μM and a maximal response at 30 μM. To determine whether the response to $H_2O_2$ was limited by its breakdown by catalase, we incubated HAEC with 5-HETE and $H_2O_2$ (100 μM) in the presence and absence of the catalase inhibitor 3-amino-1,2,4-triazole. Although this tended to increase 5-oxo-ETE synthesis somewhat (219 ± 41 vs 158 ± 24 pmol/$10^6$ cells), this difference was not statistically significant, suggesting that metabolism by catalase is not a major factor in limiting the response to $H_2O_2$ in endothelial cells. The concentration–response for *t*BuOOH, which is not a good substrate for

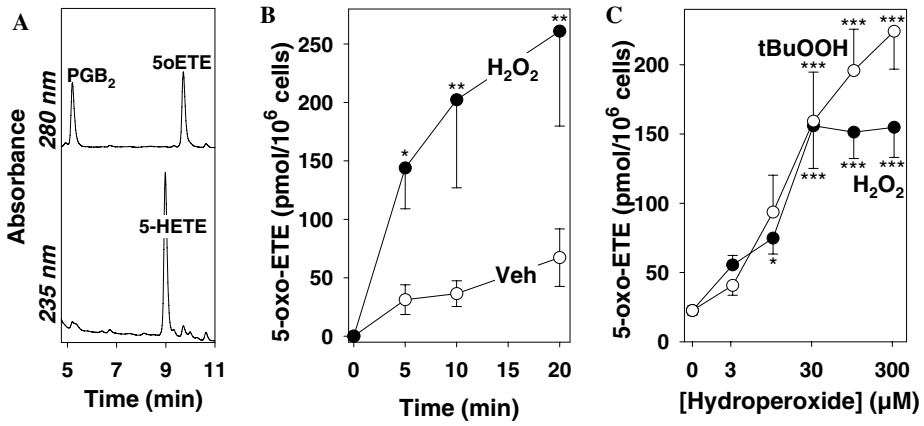


Fig. 1. Metabolism of 5-HETE by endothelial cells. (A) RP-HPLC of products formed after incubation of HAEC with 5-HETE (1 μM) for 20 min in the presence of PMS (100 μM). $PGB_2$ (100 ng) was added as an internal standard. $PGB_2$ and 5-oxo-ETE were detected at 280 nm, whereas 5-HETE was detected at 235 nm. (B) Time courses for the oxidation of 5-HETE (1 μM) to 5-oxo-ETE by HAEC in the presence of either vehicle (Veh; ○) or 100 μM $H_2O_2$ (●) ($n = 4$). (C) Concentration–response curves for the effects of $H_2O_2$ (●; $n = 5$) and *t*BuOOH (○; $n \geqslant 3$) on the synthesis of 5-oxo-ETE following incubation of HAEC with 5-HETE (1 μM) for 20 min. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

catalase, was similar to that for $H_2O_2$, except that the maximal response tended to be higher, but this was not statistically significant.

### *Involvement of the pentose phosphate pathway in regulating 5-oxo-ETE synthesis*

Oxidation of glucose-6-phosphate by the pentose phosphate pathway (PPP), which maintains NADPH in the reduced state, can be blocked by DHEA, which inhibits the initial enzyme, glucose-6-phosphate dehydrogenase [23]. To directly determine the effects of $H_2O_2$ and DHEA on $NADP^+$ in endothelial cells we measured its levels by RP-HPLC. As shown in Fig. 2A, $H_2O_2$ induces a dramatic increase in intracellular $NADP^+$ levels, which are maximal between 1 and 3 min and then decline. Preincubation with DHEA for 30 min had little effect on basal $NADP^+$ levels and did not affect its initial rate of formation. However, instead of declining after 3 min, the concentration of $NADP^+$ continued to rise up to at least 20 min in the presence of DHEA (Fig. 2A). Consistent with this, DHEA also promoted the formation of 5-oxo-ETE, which was approximately double that compared to cells treated with $H_2O_2$ alone (Fig. 2B) and was the same as that formed following treatment of cells with PMS (Fig. 3A).

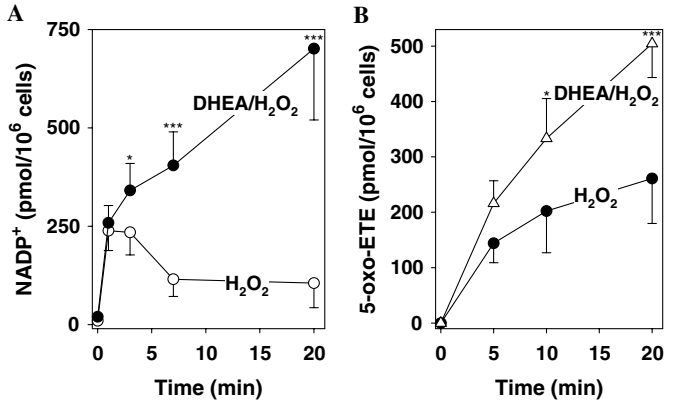


Fig. 2. Effects of DHEA on $H_2O_2$-induced changes in $NADP^+$ levels and 5-oxo-ETE synthesis. (A) Time courses for the effects of $H_2O_2$ (100 μM) on intracellular $NADP^+$ levels in HAEC following preincubation for 30 min with either vehicle (○) or 100 μM DHEA (●) ($n = 6$). (B) Time courses for 5-oxo-ETE formation induced by $H_2O_2$ following preincubation with either vehicle (●) or 100 μM DHEA (Δ) for 30 min ($n = 4$). $^{*}P < 0.05$; $^{***}P < 0.001$.

### *Role of GSH redox cycling in $H_2O_2$-induced 5-oxo-ETE synthesis*

To determine whether the GSH redox cycle is required for the stimulatory effect of $H_2O_2$ on 5-oxo-ETE formation we used a series of reagents that affect this process (Fig. 3A). Diamide, which non-enzymatically converts intracellular GSH to GSSG, had an effect almost identical to $H_2O_2$. The glutathione reductase inhibitor BCNU, which prevents the NADPH-dependent recycling of GSSG back to GSH, nearly completely blocked the effect of $H_2O_2$ on 5-oxo-ETE formation. The effect of $H_2O_2$ was also blocked by the sulfhydryl alkylating agent NEM (Fig. 3A), which nearly completely eliminated both GSH and GSSG from the cells (data not shown).

Measurement of the intracellular levels of GSH and GSSG by RP-HPLC revealed that $H_2O_2$ induced a dramatic increase in GSSG levels and a concomitant precipitous decline in GSH levels (Fig. 3B). These effects were maximal after 1 min, after which time GSSG and GSH slowly returned towards baseline values. In unstimulated cells, only $0.11 \pm 0.02\%$ of total glutathione was present in the oxidized form, whereas 1 min after treatment with $H_2O_2$, $87 \pm 2\%$ was oxidized. DHEA had no effect on the proportion of oxidized glutathione in unstimulated cells ($0.11 \pm 0.01\%$), but in its presence, $H_2O_2$ induced a greater increase in the maximal percentage of oxidized glutathione

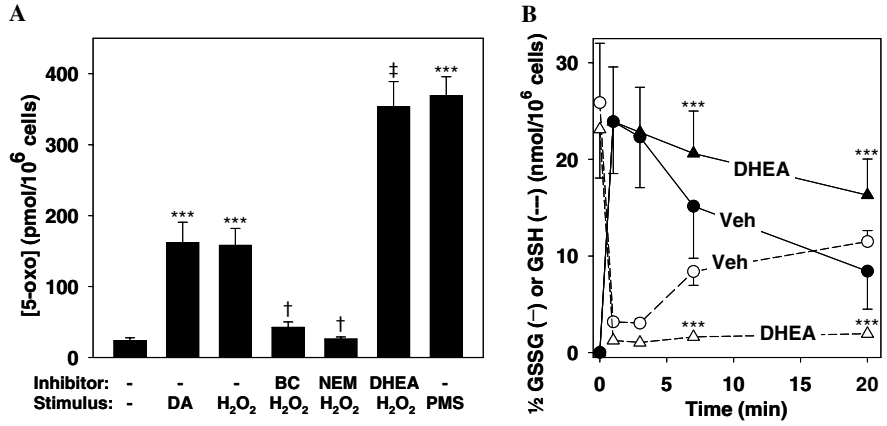


Fig. 3. Regulation of 5-oxo-ETE synthesis by the GSH redox cycle. (A) Effects of diamide (DA, 250 μM, $n = 5$), $H_2O_2$ (100 μM; $n = 6$), and PMS (100 μM; $n = 4$) on the synthesis of 5-oxo-ETE following incubation of HAEC with 5-HETE (1 μM) for 20 min. Prior to addition of 5-HETE, HAEC were preincubated for 30 min with either vehicle, BCNU (30 μM, $n = 5$) or DHEA (100 μM, $n = 5$). *** $P < 0.001$ when compared to vehicle alone; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.001$ when compared to $H_2O_2$ alone. (B) Time courses for the effects of $H_2O_2$ (100 μM) on the intracellular levels of GSH and GSSG in HAEC following preincubation for 30 min with either vehicle (●, ○) or DHEA (▲, Δ). GSH (○, Δ) and GSSG (●, ▲) were measured by RP-HPLC ($n = 6$).

($95 \pm 1\%$; $P < 0.01$) and reduced its rate of decline, so that after 20 min $89 \pm 2\%$ of total GSH remained in the oxidized form compared to $32 \pm 13\%$ in cells that had been treated with $H_2O_2$ alone. When $H_2O_2$ was added immediately after the stopping solution, the levels of GSH and GSSG were the same as in untreated controls demonstrating that $H_2O_2$-induced GSH oxidation was not due to non-enzymatic oxidation occurring after the incubations were terminated (data not shown).

## Discussion

The present study demonstrates that endothelial cells have a high capacity to metabolize 5-HETE to 5-oxo-ETE, presumably due to the presence of the $NADP^+$-dependent enzyme 5-HEDH. The activities of $NADP^+$-dependent enzymes are normally limited by the availability of this cofactor, which is present at only low levels in resting cells, as it is maintained in the reduced state, NADPH, as a means of protecting the cell against oxidative stress. This limitation can be circumvented by addition of PMS, which directly oxidizes intracellular NADPH [22], and dramatically stimulated the oxidation of 5-HETE to 5-oxo-ETE.

Oxidative stress occurs in a variety of conditions and is associated with cardiovascular disease, [24], cancer [25], and various inflammatory diseases. Endothelial cells may be subject to oxidative stress originating both from activated inflammatory cells as well as from the endothelial cells themselves [26]. Oxidative stress, in the form of $H_2O_2$ and *t*BuOOH, strongly stimulated 5-oxo-ETE formation, presumably due to their metabolism by glutathione peroxidase, coupled to the oxidation of GSH to GSSG (Fig. 4). The subsequent recycling of GSSG to GSH requires the NADPH-dependent enzyme glutathione reductase, which would produce one molecule of $NADP^+$ for every peroxide molecule reduced. This is supported by direct measurement

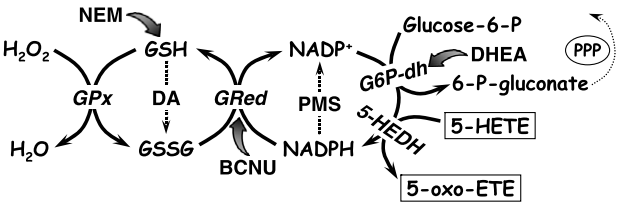


Fig. 4. Scheme for the regulation of 5-oxo-ETE synthesis by endothelial cells. Peroxides increase GSSG levels through their metabolism by glutathione peroxidase (GPx), resulting in increased $NADP^+$ levels following reduction of GSSG by glutathione reductase (GRed) and stimulation of 5-oxo-ETE synthesis. Metabolism of glucose-6-phosphate by glucose-6-phosphate dehydrogenase (G6P-dh) and other enzymes of the PPP compete with 5-HEDH for reduction of $NADP^+$, thereby limiting 5-oxo-ETE synthesis in the presence of glucose.

of GSSG and $NADP^+$, both of which increased dramatically immediately after addition of $H_2O_2$. Furthermore, depletion of cellular glutathione with NEM prevented both the increase in 5-oxo-ETE formation (Fig. 3A) and the increase in intracellular $NADP^+$ levels (data not shown) in response to $H_2O_2$. The glutathione reductase inhibitor BCNU [27] also strongly inhibited the effect of $H_2O_2$ on 5-HETE metabolism. These results suggest that endothelial cells could be a major site for the formation of 5-oxo-ETE when exposed to oxidative stress.

Glucose also appears to play a role in regulating the metabolism of 5-HETE through the oxidation of glucose-6-phosphate by the PPP. This pathway is an important component of the cell's defense against oxidative stress, as it reduces two molecules of $NADP^+$ to NADPH for every molecule of glucose-6-phosphate oxidized. We did not investigate the involvement of the PPP directly by conducting experiments in glucose-free medium, as the cells started to detach when cultured under these conditions. However, blockade of the PPP with the glucose-6-phosphate dehydrogenase inhibitor DHEA potentiated the effect of $H_2O_2$ on 5-oxo-ETE synthesis. This was accompanied by increased

intracellular levels of $NADP^+$ and the persistence of high levels of GSSG. The increased GSSG levels were presumably due to the limited availability of NADPH, which is required for the reduction of GSSG to GSH. In contrast, the basal levels of $NADP^+$ and GSSG were not affected by DHEA. These results raise the possibility that 5-oxo-ETE synthesis may be increased when endothelial cells are exposed to low glucose levels *in vivo*, as in ischemia or within inflammatory loci.

The regulation of 5-oxo-ETE formation from 5-HETE differs among cell types. In contrast to endothelial cells, oxidative stress has little or no effect on this process in neutrophils, which instead respond to activation of the respiratory burst with phorbol myristate acetate [28]. On the other hand, lymphocytes, which do not contain appreciable NADPH oxidase activity, respond only to oxidative stress with increased synthesis of 5-oxo-ETE, whereas monocytes respond to both stimuli. Although 5-HEDH activity has previously been identified only in blood cells, endothelial cells synthesize considerably more 5-oxo-ETE on a per cell basis. We previously reported that unstimulated monocytes incubated with 5-HETE (1 μM) for 5 min in the presence of glucose produced $4 \pm 1$ pmol 5-oxo-ETE/$10^6$ cells, whereas $H_2O_2$-treated monocytes produced $31 \pm 7$ pmol/$10^6$ cells [15]. Under comparable conditions, HAEC produced $28 \pm 11$ and $128 \pm 43$ pmol 5-oxo-ETE/$10^6$ cells, in the absence and presence of $H_2O_2$, respectively.

Recent genetic evidence [4] supporting a role for the 5-LO pathway in myocardial infarction and stroke is intriguing, and implicates products of this pathway in cardiovascular disease. Thus far, attention has been focused on $LTB_4$ and the cysteinyl-LTs, which act by $BLT_{1/2}$ and cys-$LT_{1/2}$ receptors, respectively [1]. The present study raises the possibility that the third arm of this pathway, 5-oxo-ETE, which acts via the OXE receptor, could also play an important role in cardiovascular disease because of its chemotactic effects on neutrophils [9] and monocytes [12]. Neutrophil infiltration appears to be an important feature of lethal ischemia-reperfusion injury [29], whereas monocytes are the precursors for macrophage-derived foam cells in atherosclerotic lesions [30]. In addition to directly stimulating these cells, 5-oxo-ETE acts synergistically with a number of peptides affecting neutrophils and monocytes, including MCP-1 [12], TNFα [31], GM-CSF [32], and RANTES [33].

In conclusion, we have shown that human endothelial cells have a high capacity to synthesize 5-oxo-ETE, especially under conditions of oxidative stress. Infiltrating leukocytes could release both oxidants, which could result in elevated intracellular levels of $NADP^+$ in endothelial cells, as well as 5-HETE, which could be converted to 5-oxo-ETE by endothelial 5-HEDH. Because of its chemoattractant properties, 5-oxo-ETE could induce further infiltration of leukocytes, resulting in prolonged or chronic inflammation. This could be exacerbated by 5-oxo-ETE-induced release of GM-CSF from monocytes [13], which could prolong the survival of granulocytes and monocytes at inflammatory loci. Thus 5-oxo-ETE may be an important mediator in chronic inflammatory diseases such as atherosclerosis and asthma, in which 5-lipoxygenase products have been demonstrated to play important roles [4,34,35].

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