Biosynthesis of hepoxilins: evidence for the presence of a hepoxilin synthase activity in rat insulinoma cells

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Abstract The 12(*S*)-lipoxygenase (12-LOX) pathway of arachidonic acid (AA) metabolism after dioxygenation to 12(*S*)-hydroperoxy-eicosatetraenoic acid is bifurcated in a reduction route to formation of 12(*S*)-hydroxy-eicosatetraenoic acid (12-HpETE) and an isomerization route to formation of hepoxilins. Interestingly, we found that the rat insulinoma RINm5F cells, which are devoid of cytoplasmic glutathione peroxidase (cGPx)/phospholipid hydroperoxide glutathione peroxidase (PHGPx), produce solely hepoxilin A_3 (HXA_3). Since HXA_3 synthesis was abolished in heat-denatured or cGPx- or PHGPx-transfected cells, it was tempting to speculate that a HXA_3 synthase activity regulated by cGPx/PHGPx is present. To confirm this assumption we incubated AA with HeLa cells overexpressing the rat leukocyte-type 12-LOX. Neither HXA_3 nor 12(*S*)-HETE were detected due to abundance of cGPx/PHGPx. But, pretreatment of transfected cells with diethyl maleate, an inhibitor of glutathione and PHGPx, restored HXA_3 synthase and 12-LOX activities. Thus, we conclude, that cells containing rat leukocyte-type 12-LOX also possess an intrinsic HXA_3 synthase activity, which is activated by inhibition of cGPx/PHGPx. In normal cells HXA_3 is down-regulated by cGPx/PHGPx, but, it is persistently activated in oxidatively stressed cells deficient in cGPx/PHGPx, such as RINm5F.

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Key words: Arachidonic acid; Cytoplasmic glutathione peroxidase; Phospholipid hydroperoxide glutathione peroxidase; Hepoxilins; Hepoxilin A_3 synthase; Leukocyte-type 12(*S*)-lipoxygenase; RINm5F cells

1. Introduction

The 12(*S*)-lipoxygenase (12-LOX) pathway of arachidonic acid (AA) metabolism after the dioxygenation to 12(*S*)-hydroperoxy-eicosatetraenoic acid (12(*S*)-HPETE) is bifurcated in a reduction route to formation of 12(*S*)-hydroxy-eicosatetraenoic acid (12(*S*)-HETE) and an isomeration route to formation of hepoxilins. Hepoxilins are hydroxy–epoxy derivates of AA, mainly observed as hepoxilin A_3 (HXA_3) (8*S*/*R*-hydroxy-11,12-epoxyeicosa-5*Z*,9*E*,14*Z*-trienoic acid) and hepoxilin B_3 (HXB_3) (10*S*/*R*-hydroxy-11,12-epoxyeicosa-5*Z*,8*Z*,14*Z*-trienoic acid) ([1] and references therein). HXA_3 is a potent biologically active lipid, which stimulates glucose-induced secretion of insulin in rat pancreatic islets, hyperpolarizes the membrane potential and controls the inhibitory postsynaptic potential in aplysia and mammalian neurons, and regulates the cell volume in platelets and aplysia neurons by activation of K^+ channels [1]. It has also been shown to augment the intracellular calcium level [2], the heat shock protein72 (HSP^{72}) [1] as well as AA release and diacylglycerol in human neutrophils [3].

AA metabolism in mammalian cells is known to be regulated by glutathione peroxidases (GPx) [4–8], which reduce the hydroperoxy fatty acids emanating from LOXs- or cyclooxygenases-catalyzed reactions to corresponding alcohols. Among various forms of GPx, two different isoforms, cGPx, the cytoplasmic isoform, and PHGPx (phospholipid hydroperoxide glutathione peroxidase), the membranous isoform, have been recently reported by us to regulate the 12-LOX-mediated eicosanoid metabolism in platelets [9]. Because GPx and hepoxilin-forming catalysts compete for the same substrate, it can be conjectured that the overall activity of glutathione peroxidases, or in other words the overall hydroperoxide tone, determines the rate of hepoxilin formation. Consequently, inhibition of both cGPx and PHGPx by iodoacetate has been shown to enhance the synthesis of hepoxilins with concomitant reduction of 12(*S*)-HETE production [9]. This indicated a shift in conversion of 12(*S*)-HPETE from the reduction pathway to the so-called isomerization pathway. Although PHGPx activity contributes only 2% to the total 12(*S*)-HPETE reductase activity (mostly cGPx activity) its share in reducing the 12(*S*)-HPETE is far more pronounced as found in cGPx-depleted selenium-deficient UT7 cells [9] and cGPx knock-out mice [10].

The biosynthesis of hepoxilins by isomerization pathway is highly controversial. In order to elucidate the mechanism Dr. Pace-Asciak's group performed a number of mechanistic studies during the last decade and proposed an enzymatic pathway for the formation of hepoxilins. His proposal was based on the stereoselective conversion of 12(*S*)-HPETE into 8(*S*)- and 8(*R*)-epimers of HXA_3 by rat pineal gland [11], while 12(*R*)-HPETE remained unmetabolized. Moreover, the conversion

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Abbreviations: DEM, diethyl maleate; cGPx, cytoplasmic glutathione peroxidase; PHGPx, phospholipid hydroperoxide glutathione peroxidase; GSH, glutathione; 12(*S*)-HETE, 12(*S*)-hydroxy-eicosatetraenoic acid; 12(*S*)-HPETE, 12(*S*)-hydroperoxy-eicosatetraenoic acid; HXA_3, hepoxilin A_3 or 8*S*/*R*-hydroxy-11,12-epoxyeicosa-5*Z*,9*E*,14*Z*-trienoic acid; HXB_3, hepoxilin B_3 or 10*S*/*R*-hydroxy-11,12-epoxyeicosa-5*Z*,8*Z*,14*Z*-trienoic acid; 12-LOX, 12(*S*)-lipoxygenase; Me-TMS, methyl-trimethylsilyl; $TrXA_3$, trioxilin A_3

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of AA by the pineal gland resulted in the formation of HXA_3 of the native 11(*S*),12(*S*)-configuration of epoxide, showing the presence of 12-LOX in the pineal tissue [12], which led to the conclusion that the biosynthesis of hepoxilins is only possible in cells and tissues that contain 12-LOX. However, a clear-cut evidence for the presence of HXA_3 synthase activity is not yet presented. In the present study, we have shown that RINm5F rat insulinoma cells contain a heat-sensitive leukocyte-type 12-LOX enzyme, which expresses HXA_3 synthase activity and is capable of transforming AA or 12(*S*)-HPETE selectively to HXA_3. Furthermore, this activity is regulated by selenoenzymes, e.g. cGPx and PHGPx. To prove the regulation by selenoenzymes, we used native RINm5F cells, which are devoid of cGPx and PHGPx [13] and produce large amounts of HXA_3 when incubated with AA. The HXA_3 synthesis was however abolished when cells stably transfected with cGPx or PHGPx were used.

2. Materials and methods

2.1. Cell culture

RINm5F, rat insulinoma cells (ATCC, Washington, DC, USA), were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The cells were grown in humidified incubators at 37°C with 5% CO_2. RINm5F cells stably transfected with cGPx, a kind gift from Dr. Tiedge, Hannover, Germany, were cultured in the same culture medium as above, but supplemented with 400 μg/ml geneticin (Invitrogen) and 10 nM sodium selenite.

2.2. Assay of HXA_3 synthase activity

The hepoxilin synthase A_3 activity was measured by incubating the lysate from RINm5F cells with 100 μM AA in a volume of 200 μl for 30 min at 37°C and measuring the formation of hepoxilins using GC-MS or HPLC. The reaction was stopped by acidification with 1 N HCl to pH 3.0. The reaction mixture was allowed to incubate at room temperature in acidic conditions for 15 min before extracting the lipids twice with 3 volumes of ethyl acetate. The lipid fractions were extracted with ethyl acetate, evaporated to dryness under nitrogen, reconstituted in *n*-hexane and analyzed with gas chromatography-mass spectrometry (GC-MS).

2.3. GC-MS

The lipids were converted to their methyl esters by the addition of 300 μl ethereal diazomethane for 5 min. Thereafter the samples were evaporated to dryness, 30 μl *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide was added and the mixture was derivatized by heating at 60°C for 30 min. GC-MS was performed by means of a Varian Saturn 4D GC-MS-MS system equipped with a Supelco DB5-MS column (30 m×0.25 mm; 0.25 μM d_f). The temperature program was started at 150°C increasing to 250°C within 10 min with a rate of 10°C/min. The temperature of injector and transfer line were 230°C and 220°C respectively.

2.4. Detection of HXA_3 in RINm5F cells by HPLC

RINm5F cells (10×10^6 cells) were harvested and resuspended in 100 μl 0.1 M sodium phosphate buffer (pH 7.4). 100 μM AA was added and the reaction was allowed to proceed at 37°C for 30 min. The reaction was stopped and the lipids were extracted by the addition of ether–methanol–1 M citrate (135:15:1, v/v). The organic phase was evaporated and the residue was taken into 100 μl ethyl acetate and derivatized by the addition of 9-anthryldiazomethane (100 μg/reaction) [11]. The reaction mixture was stirred at room temperature for 1 h. The ethyl acetate was then evaporated, the residue was reconstituted in the solvent system and the lipids were loaded onto a Novapk C_{18} (250×46 mm; 5 μm particle size) reverse phase column. Acetonitrile–methanol–water (90:6:4 v/v) was used as mobile phase. The eluate was UV-monitored using a Shimadzu diode-array detector set at 254 nm and fluorescence-monitored (excitation 254 nm, emission 400 nm) using a Shimadzu fluorescence detector.

3. Results

3.1. RINm5F cells express hepoxilin synthase-like activity

Cultured RINm5F cells were incubated with 100 μM AA for 20 min and the products were analyzed as ADAM ester derivatives by HPLC monitoring fluorescence detection (see Section 2). As shown in Fig. 1A, a distinct HXA_3 peak was observed, which appeared at the same retention time as the authentic standard (panel III). Upon longer incubation, e.g. 40 min, HXA_3 peak was substantially reduced and a more polar peak appeared (not shown), which has been earlier shown to characterize the hydrolysis products of HXA_3, i.e. 8(*S*/*R*),11(*R*),12(*S*)-trihydroxy-eicosa-5*Z*,9*E*,14*Z*-trienoic acid and 10(*S*/*R*),11(*R*),12(*R*)-trihydroxy-eicosa-5*Z*,8*Z*,14*Z*-trienoic acid, trivially known as trioxilins A_3 ($TrXA_3$) [1]. As these hepoxilins are hydrolyzed by cellular epoxide hydrolases, HXA_3 formation was determined in RINm5F cells pretreated with 100 μg/ml trichloro-propylene oxide (TCPO), an inhibitor of epoxide hydrolases. After 40 min incubation with AA a significant increase in HXA_3 formation was observed

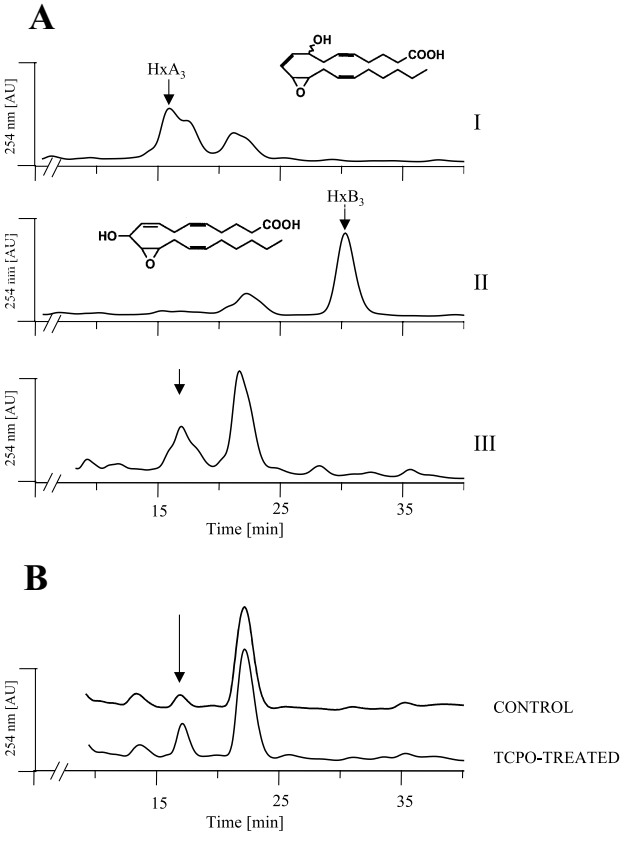

Fig. 1. Representative profiles of UV-detectable synthesis of HXA_3 untreated (A) and TCPO-treated RINm5F cells. A: Native RINm5F cells were incubated with 100 μM AA at 37°C for 30 min. The lipids were extracted by organic solvents and derivatized by the addition of 100 μg 9-anthryldiazomethane. The lipids were separated by RP-HPLC and detected at 254 nm. Panels I and II represent HXA_3 and HXB_3 standard, respectively. Panel III represents the formation of HXA_3 alone from the reaction of AA with RINm5F cells. Peak next to HXA_3 is not related to AA metabolism via 12-LOX pathway. B: RINm5F cells with or without pretreatment of 10 μM TCPO, an epoxide hydrolase inhibitor, were incubated with 100 μM AA and the samples were processed as described in (A). Significant increase in HXA_3 formation can be seen in TCPO-treated cells.

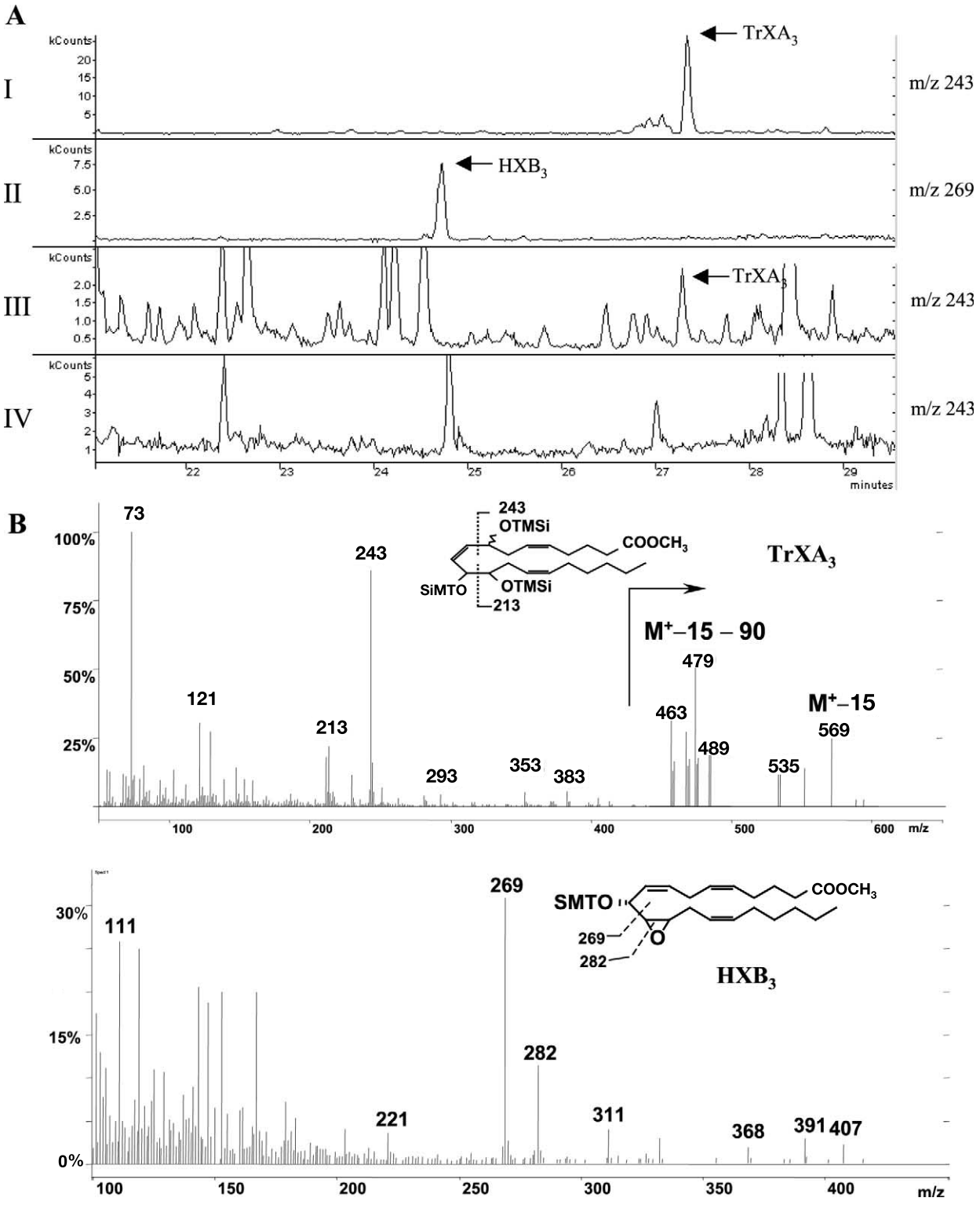

Fig. 2. Representative mass chromatograms ($[M]^+$) of Me-TMS derivative of $TrXA_3$ obtained from the transformation of AA by RINm5F cells. Incubation of RINm5F cells with AA were carried out as described in Fig. 1A and Me-TMS derivatives were analyzed by GC-MS (see Section 2). A: Panels I and II show the representative mass chromatograms of Me-TMS derivative of standards $TrXA_3$ (*m/z* 243) and HXB_3 (*m/z* 269), respectively. Panel III shows the representative mass chromatogram of Me-TMS derivative of $TrXA_3$ obtained from the transformation of AA by native RINm5F cells. Panel IV shows the abrogation of $TrXA_3$ by scanning the mass chromatogram of Me-TMS derivative of $TrXA_3$ obtained from the reaction of AA with heat-denatured RINm5F cells. B: Electron impact mass spectra of the Me-TMS derivatives of $TrXA_3$ and HXB_3 showing characteristic ions *m/z* 243 and *m/z* 269, respectively.

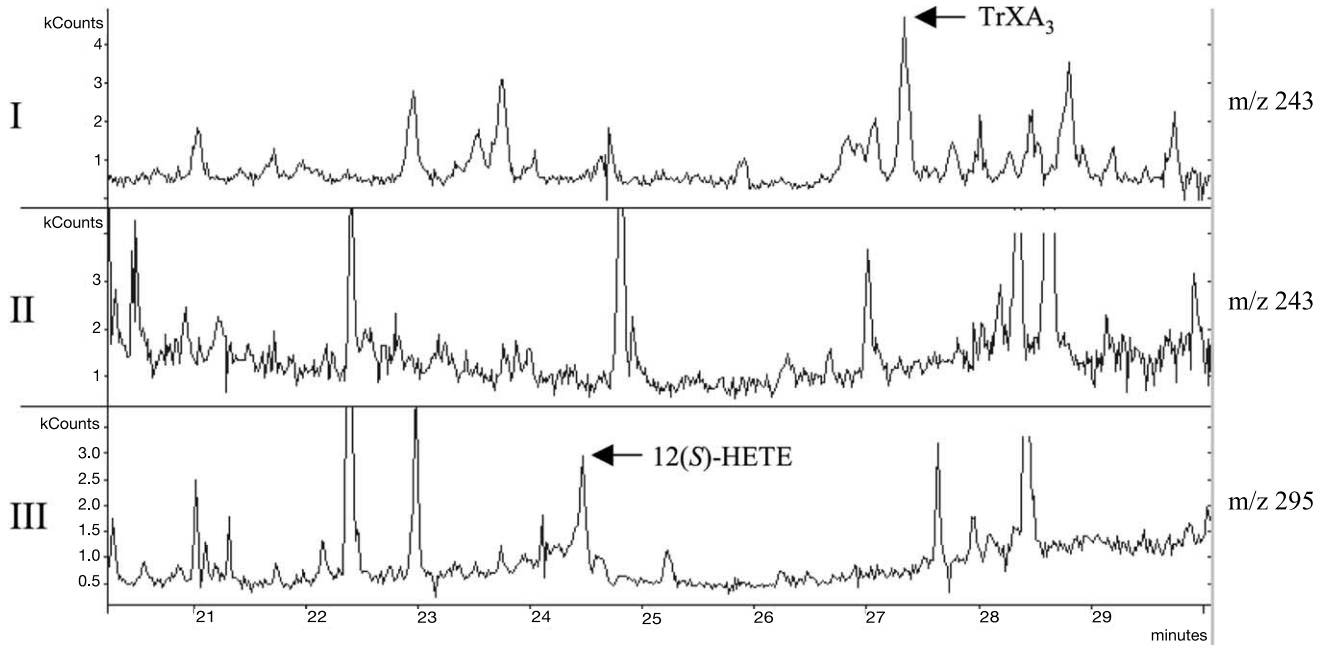

Fig. 3. Representative mass chromatograms ($[M]^+$) of Me-TMS derivative of $TrXA_3$ and 12(*S*)-HETE obtained from the transformation of AA by native and cGPx-transfected RINm5F cells. Panel I shows the representative mass chromatogram of Me-TMS derivative of $TrXA_3$ (*m/z* 243) obtained from the transformation of AA by native RINm5F cells. Panel II shows the abrogation of $TrXA_3$ by scanning the mass chromatogram of Me-TMS derivative of $TrXA_3$ obtained from the reaction of AA with cGPx-transfected RINm5F cells. Panel III shows the representative mass chromatogram (*m/z* 295) of Me-TMS derivative of 12(*S*)-HETE formed from the reaction of AA with cGPx-transfected RINm5F cells.

(Fig. 1B) without appearance of a polar peak. To confirm the chemical structure of HXA_3 from RINm5F cells, the incubation mixture was extracted as described in Section 2, the residue acid-hydrolyzed (to transform HXA_3 to stable $TrXA_3$) and converted to methyl-trimethylsilyl (Me-TMS) derivatives for GC-MS analysis. As shown in Fig. 2 (panel III), a singular peak characterizing $TrXA_3$ was observed, but no HXB_3 was detected, indicating the absence of heme-catalyzed reaction.

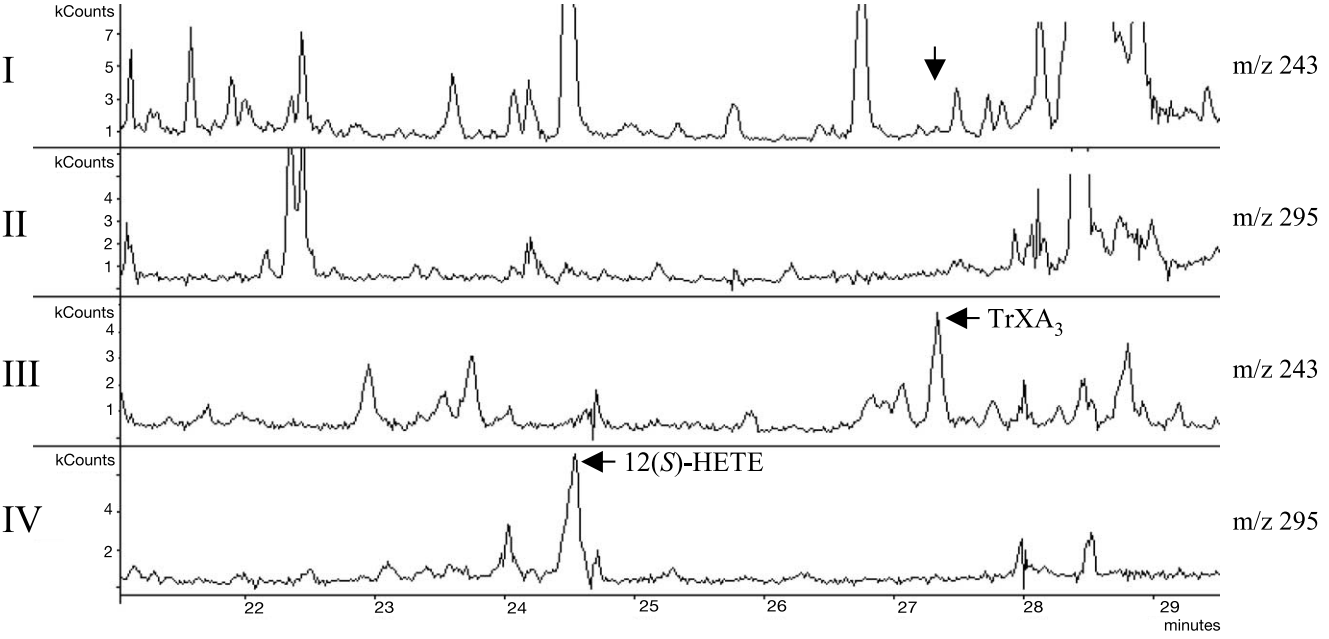

Fig. 4. Production of HXA_3 from the transformation of AA by HeLa cells transfected with rat leukocyte-type 12-LOX-cDNA. Panels I and II show the abrogation of $TrXA_3$ as well as 12(*S*)-HETE by scanning the mass chromatogram of Me-TMS derivatives of $TrXA_3$ (*m/z* 243) and 12(*S*)-HETE (*m/z* 295), respectively, obtained from the reaction of AA with HeLa cells transfected with rat leukocyte-type 12-LOX. Panels III and IV show the effect of inhibition of GSH and PHGPx by 2 mM DEM on the formation of $TrXA_3$ as well as 12(*S*)-HETE, respectively. Representative mass chromatograms of Me-TMS derivatives of $TrXA_3$ (*m/z* 243) and 12(*S*)-HETE (*m/z* 295), show clearly the formation of HXA_3 as well as 12(*S*)-HETE by transfected HeLa cells.

Strikingly, the heat-denatured (90°C for 10 min) cell lysate failed to produce any HXA_3, pinpointing the enzymatic pathway of its formation (Fig. 2, panel IV). The absence of 12-LOX activity as well (not shown) supported the earlier assumption by Pace-Asciak's group that the HXA_3 synthase activity is only present in cells that contain 12-LOX activity [12].

3.2. GPx inhibit the formation of HXA_3

Recently, we showed the prominent role of PHGPx as a 12(*S*)-HPETE reductase in human platelets [9]. In absence of cGPx the reduction 12(*S*)-HPETE to 12(*S*)-HETE was taken over by PHGPx. But, inactivation of both selenoenzymes cGPx and PHGPx by iodoacetate led to accumulation of 12(*S*)-HPETE, the metabolism of which was consequently diverted to HXA_3 and HXB_3 formation. Since RINm5F cells are almost devoid of cGPx and PHGPx [13], as expected these cells synthesized HXA_3 when incubated with AA as substrate (Fig. 3, panel I). Conversely, we assumed that the presence of GPx should diminish the formation of HXA_3 (RINm5F cells do not produce HXB_3!). Indeed, upon incubation with AA cultured RINm5F cells, stably transfected with cGPx did not produce any HXA_3 as determined by GC-MS (Fig. 3, panel II). However, due to the down-regulation of 12-LOX by cGPx very little 12(*S*)-HETE was detectable (Fig. 3, panel III). Incubation with 12(*S*)-HPETE of native or cGPx-transfected RINm5F cells reproduced identical results as with AA (not shown). Similar results, albeit less pronounced, were obtained with PHGPx-transfected RINm5F cells (not shown).

3.3. Rat leukocyte-type 12-LOX possesses intrinsic HXA_3 synthase-like activity

To investigate if this regulatory mechanism by GPx of 12-LOX possessing intrinsic HXA_3 synthase activity is a common phenomenon in other cells, we transfected rat leukocyte-type 12-LOX-cDNA in HeLa cells, which show low expression of 12-LOX. Upon incubation of cultured transfected cells with AA, neither 12(*S*)-HETE nor HXA_3 was produced, suggesting the complete down-regulation of 12-LOX by selenoenzymes abundantly present in the cell (Fig. 4, panels I and II). However, pretreatment of 12-LOX-transfected cells with 2 mM diethyl maleate (DEM), which depletes cellular glutathione (GSH) and thus inhibits PHGPx [7], caused significant enhancement of HXA_3 synthesis (Fig. 4, panel III), but also a little formation of 12(*S*)-HETE. As no HXB_3 formation was detected in GSH- and GPx-depleted cells, an up-regulation of HXA_3 synthase activity seems to be evident.

4. Discussion

Lipoxygenases are bifunctional enzymes exhibiting both fatty acid dioxygenase and lipohydroperoxidase activities. The lipohydroperoxidase activity, unlike the dioxygenase activity, does not require oxygen insertion. Among various reports on the enzymatic conversion of AA with different lipoxygenases, it has been, for instance, clearly demonstrated that both soybean lipoxygenase-1 [14] and reticulocyte-type 15-lipoxygenase [15] are capable of converting hydroperoxy-polyenoic fatty acids to their hydroxy–epoxy derivatives. Thus, 15-LOX has been shown to convert 15-hydroperoxy fatty acids and AA to epoxy-leukotrienes [15,16]. Also, 15-LOX from garlic roots converted AA, but not 12(*S*)-HPETE, to 14,15-hepoxilins [17]. Although platelet-type 12-LOX does not possess any lipohydroperoxidase activity, Bryant et al. showed in the lysate from selenium-deficient platelets the formation of hydroxy–epoxy derivatives of AA, currently known as $TrXA_3$ and $TrXB_3$ [18]. Later, using hemin and hemoglobin Pace-Asciak et al. demonstrated that the isomerization of 12(*S*)-HPETE to HXA_3 and HXB_3 is heme-catalyzed [19]. Recently, our laboratory also found that the conversion of AA to HXA_3 and HXB_3 in platelets, as reported by Bryant et al. [8], is heme- and not enzyme-catalyzed, and that cGPx and PHGPx prevent the detection of hepoxilins by diverting the 12(*S*)-HPETE → hepoxilin pathway to 12(*S*)-HPETE → 12(*S*)-HETE pathway [9], thus revealing the importance of selenoenzymes in the regulatory network of AA metabolism.

RINm5F rat insulinoma cells, which possess leukocyte-type 12-LOX and are devoid of cGPx and PHGPx [13], produced only HXA_3 when incubated with AA (Fig. 1A). Moreover, no HXA_3 was detected in the lysate from heat-inactivated cells (Fig. 2A, panel IV). But, cultured cells stably transfected with cGPx or PHGPx-cDNA did not show any formation of HXA_3 (Fig. 3, panel II). These results pinpointed the presence of HXA_3 synthase-like activity in RINm5F cells and its regulation by GPx. Strikingly, 12-LOX activity was also absent in heat-denatured RINm5F cells (not shown), which supported the assumption of Pace-Asciak's group that the enzymatic formation of HXA_3 requires inevitably the presence of 12-LOX enzyme [20]. The fact that HeLa cells overexpressing rat leukocyte-type 12-LOX did not produce any HXA_3 and 12(*S*)-HETE under normal conditions, suggests the complete down-regulation of 12-LOX activity by the abundantly present cGPx and PHGPx. But, drastic increase in the formation of HXA_3 upon treatment with DEM, which depletes cellular GSH and inhibits PHGPx thus elevating the overall hydroperoxide tone, added support to our assumption that the rat leukocyte-type 12-LOX possesses an intrinsic HXA_3 synthase activity. The latter was found to be finely tuned by cellular GPx cGPx and PHGPx. This regulation by GPx is in line with earlier observations reported for 5-, 12- and 15-LOX [4,6,8].

Normally, in various cell types the presence of GPx exerts the primacy of the reduction pathway over the hepoxilin pathway. In our experiments with HeLa cells overexpressing the rat leukocyte-type 12-LOX, selenoenzymes obviously diminished the cellular peroxide tone to such an extent that even a minimum peroxide level essential to trigger the activation of 12-LOX may not be present. Thus, an important message can be deduced from the above observations, that in most cell types under physiological conditions abundantly present selenoenzymes prevent the hepoxilin formation and divert the AA metabolism to the formation of 12(*S*)-HETE from intermediate 12(*S*)-HPETE. This means de facto that the formation of HXA_3 is only restricted to pathological cases, say to diseases with persistent oxidative stress, in which deficiency of selenoenzymes is prevalent. It should however be stressed that the mechanisms underlying the activation of HXA_3 synthase activity are primarily associated with GSH depletion and inhibition of GPx rather than with increase in reactive oxygen species. Since HXA_3 is know to be cell protective [1], its formation can be regarded as a counteraction to the permanent oxidative-stressed cellular status. Thus, synthesis of hepoxilins plays an important role in the processing of cellular hydro-

peroxides and hence in overall regulation of the 12-LOX pathway.

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