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HEPOXILIN A_3 INDUCES HEAT SHOCK PROTEIN (HSP^{72}) EXPRESSION IN HUMAN NEUTROPHILS

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In this paper we show that hepoxilin A_3 induces the expression of heat shock protein expression in human neutrophils at a concentration of 100 nM using Western blotting techniques employing the use of a commercial monoclonal antibody to HSP^{72}. No regiospecificity was observed as the 8S enantiomer of HxA_3 was as active as the 8R enantiomer of HxA_3. Comparison of the effects of HxA_3 with 12S-HETE and PGA_1 indicated that HxA_3 was as effective as 12S-HETE although PGA_1 was essentially inactive at the same concentration used for these 12-lipoxygenase products.

The response of cells to elevated temperatures, the so-called heat shock response, is associated with induction in the transcription of mRNAs encoding a family of proteins called 'heat shock proteins' (1, 2). The expression of this family of proteins has been associated with cell injury and with the repair process (3). Hence heat shock proteins have been referred to as molecular chaperones, in that they are believed to be involved in the folding and refolding of proteins to prevent their retention in the endoplasmic reticulum (4). Recently it was shown that the heat shock proteins are also induced in human leukocytes during infection and inflammation suggesting that HSPs may also serve a role in the processes of inflammation (5). Conversely, heat shock stimulates the release of arachidonic acid and the subsequent formation of prostaglandins and leukotriene B_4 (6). Indeed 12S-HETE, one of the products derived from the 12-lipoxygenase pathway, which has chemoattractant properties to neutrophils (7, 8), has recently been reported to be formed by human leukocytes (9) and to induce the expression of HSP^{72} (10). Since the hepoxilins are also products of the 12-lipoxygenase pathway (11, 12, 13) and have been shown to be formed by neutrophils (14) and to have actions on calcium mobilization (14) and second

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Abbreviations: Hepoxilin A_3, HxA_3, 8 (R or S) -hydroxy-11,12-epoxyeicosa-5Z, 9E, 14Z-trienoic acid; Trioxilin A_3, $TrXA_3$, 8 (S), 11 (R), 12 (S)-trihydroxy-eicosa-5Z, 9E, 14Z-trienoic acid; Hepoxilin A_3-C, HxA_3-C, 8 (R or S), 12 (S) -dihydroxy - 11 (R) - glutathionyl-eicosa-5Z, 9E, 14Z-trienoic acid; 12S-HETE, 12 (S)-hydroxy-eicosa-5Z, 8Z, 10E, 14Z-trienoic acid; HSP^{72}, heat shock $protein^{72}$; PG, prostaglandin; Anti-HSP^{72}, monoclonal antibody to HSP^{72}.

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messenger release (15), we were interested in determining whether these products share with 12S-HETE the capacity of stimulating HSP^{72} expression.

The data presented herein demonstrate new biological actions of hepoxilins. We and others have previously shown that these compounds may act as neuromodulators (16-19) as well as inflammatory modulators in the rat skin (20, 21). They appear to have as their basis the modulation of intracellular calcium in the cell (14, 22), although their ability to increase the release of arachidonic acid and diacylglycerol in the neutrophil occurs at concentrations well below their ability to modify intracellular calcium (15). We have also recently shown that the hepoxilins, more specifically the 8S-enantiomer, although not capable of constricting aortic strips in vitro, is capable of potentiating at 10^{-8}M concentrations the contraction of these vascular strips to norepinephrine (23). Hence the hepoxilins appear to modulate the actions of vasoconstrictor hormones.

The present study was carried out to investigate the ability of the 8R and 8S enantiomers of HxA_3 to induce HSP expression as monitored with a monoclonal antibody to HSP^{72} and to compare the potency of the hepoxilins with that of other eicosanoids shown to have this effect, namely 12S-HETE (10) and PGA_1 (24, 25).

MATERIALS AND METHODS

Materials: HxA_3 (8R and 8S enantiomers) were kindly provided by Dr. E.J. Corey (Harvard University) and dissolved in ethanol. PGA_1 was purchased from Sigma (St. Louis, MO) and 12S-HETE was obtained from Cayman Chemical Co. (Ann Arbor, MI). All compounds were dissolved in ethanol and added to the cells in 5µl of ethanol. Anti-HSP^{72} monoclonal antibody (dilution 1:1000), rabbit anti-mouse IgG antibody (dilution 1:1000) and [^{125}I]-labeled protein A (specific activity 14.5 mCi/µg iodine) were purchased from Amersham Canada (Toronto).

Solutions: Sodium bicarbonate-free RPMI 1640 with L-glutamine and 25mM HEPES was buffered to pH 7.2 using 1N HCl and used as incubation medium. Phosphate-buffered saline (PBS) contained in mM: NaCl (120), Na_2PO_4 (10) and $NaHPO_4$ (3), pH adjusted to 7.4. Lysis buffer was made up of Tris.HCl (10mM) and EDTA (1mM), pH 7.5.

Preparation of neutrophils: Neutrophils were separated from fresh human venous blood by dextran sedimentation followed by centrifugation on a Ficoll-Hypaque gradient (27). Residual red blood cells were removed by lysis with NH_4Cl. Washed cells were maintained at room temperature in HEPES buffered RPMI at 10^7 cells/ml until use, normally within 30 min of preparation. Cells were counted on a Coulter cell counter and were shown to have >95% viability through trypan blue dye exclusion.

Incubation of cells: Freshly isolated neutrophils (1 x 10^7 cells/500µl RPMI 1640) were incubated with the drugs (20 and 40µg in 5µl ethanol) or with vehicle alone (5µl ethanol) in a water bath at 37°C for 60 min with shaking. Neutrophils were also heat shocked by heating at 42°C for 60 min. After incubation, samples were placed on ice to stop the reaction.

Protein Extraction and Western Blotting: Cytosolic proteins were extracted by centrifuging the cells at 1000 rpm for 5 min at room temperature. The pellet was washed with 1ml of ice-cold PBS and 2-3 vols. of ice-cold lysis buffer was added. Cells were broken by passing through a 25G needle fifteen times. Cell lysates were microcentrifuged at 16,000 rpm for 30 min at 4°C. Supernatants were transferred to clean tubes and assayed for protein concentration using the Bio-Rad method (28). The same amount of protein (12.5µg) from each sample was loaded on a 12.5% SDS-polyacrylamide gel (BioRad mini gel) and resolved at 150 volts for 45min. Proteins were transferred to a nitrocellulose membrane overnight at 4°C at 30 volts. Nonspecific antibody sites on the nitrocellulose were blocked with 3% gelatin-TBS. HSP^{70}-group proteins were detected with the monoclonal anti-72kD heat shock protein antibody. The bound monoclonal

antibodies were detected using rabbit anti-mouse IgG antibody and subsequently with [^{125}I]-labeled protein A. The membrane was exposed overnight to X-ray film and the density of the bands was quantified using densitometry.

RESULTS AND DISCUSSION

Human neutrophils responded to heat shock treatment or various eicosanoids by expressing HSP^{72}. Figure 1 shows a Western blot of an experiment comparing the efficacy of HSP^{72} protein induction by heat shock treatment with that observed by treatment with 12S-HETE or 8S-HxA_3 within the concentration range of 100-200nM. This data demonstrates that the hepoxilins are capable of inducing HSP expression at about the same concentration range as 12S-HETE. Quantitatively, the effects of 12S-HETE and HxA_3 are not significantly different from the heat shock response. Quantification of the results of this and other experiments are shown in Figure 2. Here, it can be seen that both 12S-HETE and HxA_3 have similar potency within the range of concentration used (100 - 200 nM). It can also be seen that PGA_1 is ineffective in inducing HSP^{72} expression. In additional studies (not shown) it was determined that the 8R enantiomer of HxA_3 was as effective as 8S-HxA_3 . This is in sharp contrast to our recent observations that only the 8R-HxA_3 was effective in causing vascular permeability in the rat skin (20). The latter preparation may be more under receptor control than the expression of HSP^{72} as seen in this study. Our present results are expressed as percent of control to minimise interexperiment variability although significant 'spread' in data might reflect individual variation in neutrophils as blood from two different individuals was used on three different experiment days. Neutrophils also respond differently and more effectively to the hepoxilins than human or mouse neuroblastoma cells in that the latter cells require μg amounts of PGA_1 and hepoxilins to induce the expression of HSP^{72} (unpublished observations). Data published by Santoro et al also has indicated that μg amounts of PGs are needed for neuroblastoma HSP to be induced (25).

The findings that hepoxilins and 12S-HETE (but not PGA_1) induce expression of HSP suggest that these 12-lipoxygenase products may represent natural regulators of cellular repair and immune processes. Damage to cells is accompanied by a rapid release of arachidonic acid, the precursor to the hepoxilins and 12-HETE. If, indeed this precursor is rapidly transformed via 12-

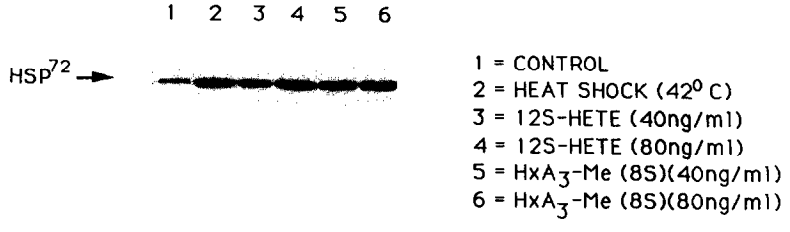

Figure 1. Western blot. Human neutrophils (1x10^7 cells/500 μl RPMI 1640) were incubated with vehicle or the various drugs in ethanol, for 60 min at 37°C, or for 42°C (heat shock) in the absence of drugs. Proteins were extracted after cell lysis as described in Methods. Equivalent amount of protein from each sample was separated on SDS-polyacrylamide gel and transferred to nitrocellulose membrane. HSP^{72} was detected with a monoclonal anti HSP^{72} antibody.

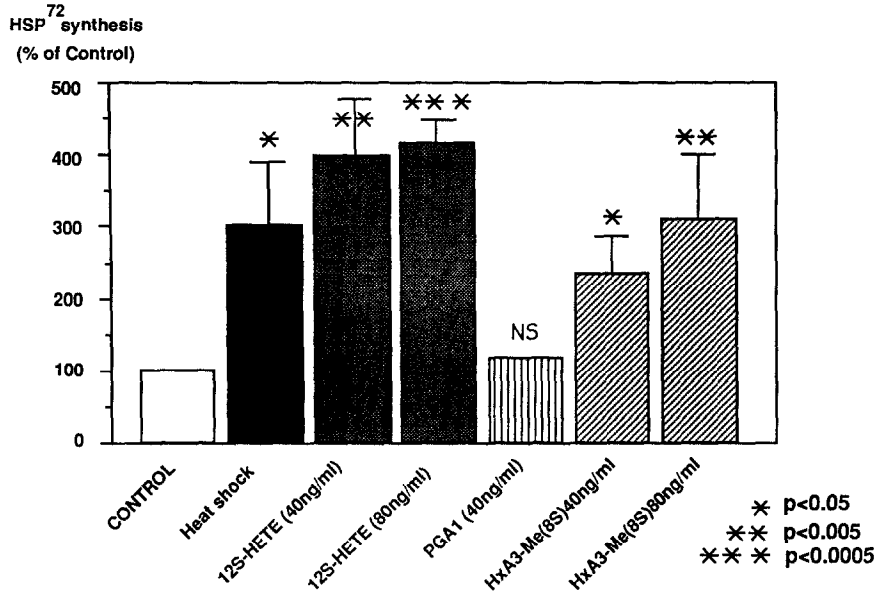

Figure 2. Quantitative data from a number of separate experiments (n=3 separate donors) showing the effect of heat shock (42°C), 12S-HETE (40 and 80 ng/ml), PGA_1 (40 ng/ml) and 8S-HxA_3 (40 and 80 ng/ml) on the induction of HSP^{72} in human neutrophils as determined by Western blotting techniques. Data is similar to that in Figure 1 except that this Figure shows the results of a number of experiments. Data shown is after quantitation of the radioactive spots on the X-ray film by densitometry. Significance is calculated relative to control values using Student's t-test.

lipoxygenase as is expected during cell damage, these compounds may in turn act to regulate the expression of HSP which may be involved in the repair process. It would be of interest to investigate whether the temporal formation of these 12-lipoxygenase products precedes HSP expression and whether inhibition of the expression of 12-lipoxygenase results in an interruption of HSP expression and the resulting repair process in damaged tissue.

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