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Modification of platelet proteins by 4-hydroxynonenal: Potential Mechanisms for inhibition of aggregation and metabolism

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ABSTRACT

Platelet aggregation is an essential response to tissue injury and is associated with activation of pro-oxidant enzymes, such as cyclooxygenase, and is also a highly energetic process. The two central energetic pathways in the cell, glycolysis and mitochondrial oxidative phosphorylation, are susceptible to damage by reactive lipid species. Interestingly, how platelet metabolism is affected by the oxidative stress associated with aggregation is largely unexplored. To address this issue, we examined the response of human platelets to 4-hydroxynonenal (4-HNE), a reactive lipid species which is generated during thrombus formation and during oxidative stress. Elevated plasma 4-HNE has been associated with renal failure, septic shock and cardiopulmonary bypass surgery. In this study, we found that 4-HNE decreased thrombin stimulated platelet aggregation by approximately 60%. The metabolomics analysis demonstrated that underlying our previous observation of a stimulation of platelet energetics by thrombin glycolysis and TCA (Tricarboxylic acid) metabolites were increased. Next, we assessed the effect of both 4-HNE and alkyne HNE (A-HNE) on bioenergetics and targeted metabolomics, and found a stimulatory effect on glycolysis, associated with inhibition of bioenergetic parameters. In the presence of HNE and thrombin glycolysis was further stimulated but the levels of the TCA metabolites were markedly suppressed. Identification of proteins modified by A-HNE followed by click chemistry and mass spectrometry revealed essential targets in platelet activation including proteins involved in metabolism, adhesion, cytoskeletal reorganization, aggregation, vesicular transport, protein folding, antioxidant proteins, and small GTPases. In summary, the biological effects of 4-HNE can be more effectively explained in platelets by the integrated effects of the modification of an electrophile responsive proteome rather than the isolated effects of candidate proteins.

1. Introduction

Lipid peroxidation can produce reactive electrophiles such as 4-hydroxy-2-nonenal (4-HNE), which in turn can alter cellular function by forming stable adducts with proteins [1-4]. 4-HNE has been identified in human atherosclerotic lesions, brain sections of patients with Alzheimer's and Parkinson's disease, kidney tissue of patients with renal cell carcinoma and diabetic nephropathy and placentas of women with preeclampsia [5-9]. Based on these studies, 4-HNE has been considered a negative prognostic marker and mediator of oxidative stress in a broad range of diseases. Conversely, some studies have reported that low concentrations of 4-HNE can have a protective role against oxidative stress through activation of Nrf2 and the induction of antioxidant enzymes such as heme oxygenase 1 (HO-1) and glutathione-S-transferase [10-12]. It has been shown that 4-HNE forms adducts with mitochondrial and glycolytic proteins, such as cytochrome c_1 (complex III), cytochrome *c*, electron transfer flavoprotein alpha, glyceraldehyde-3-phosphate dehydrogenase,

Abbreviations: 4-HNE, 4-hydroxynonenal; AA, antimycin A; A-HNE, alkyne HNE; ECAR, extracellular acidification rate; LDH, lactate dehydrogenase; MnSOD, manganese superoxide dismutase; OCR, oxygen consumption rate; PRP, platelet rich plasma; vWF, von Willebrand Factor; XF, Extracellular Flux; TCA, tricarboxylic acid cycle

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aldolase A, and inhibits the activity of these metabolic proteins [13-15].

The platelet offers an interesting and biologically relevant setting in which to assess the impact of 4-HNE, and how identification of protein adducts is linked to changes in platelet function to mechanism. This is important because previous studies have largely taken a candidate target approach to identifying mechanisms of 4-HNE dependent changes in function. It is now becoming clear that the biological effects of reactive electrophiles are mediated through the generation of an electrophile responsive proteome which has the potential to impact biological function through the cumulative effects at multiple targets [16,17]. In the platelet, we can identify both effects on bioenergetics and platelet function and can link these to the protein targets so providing an ideal context to test these concepts.

Previous studies have shown that 4-HNE can affect platelet aggregation and possibly act as a negative feedback modulator of platelet function. However, the data presented in the literature are not consistent. For example, using platelet rich plasma (PRP) from healthy human volunteers it was reported that 4-HNE at high concentrations (0.5–2 mM) did not affect thrombin-induced (2 U/ml) aggregation [18]. However, the authors reported an approximately a 50% decrease in ADP stimulated aggregation of PRP when pre-treated with 4-HNE (330 μM) for 10 min [18]. Conversely, low concentrations of 4-HNE were more potent in inhibiting thrombin (0.5 U/ml) mediated aggregation in washed platelets [18]. In contrast, another study reported that incubation of healthy PRP with HNE for 1 min caused a significant potentiation of aggregation induced by thrombin (0.02 U/ml) and ADP, but not collagen when compared to control conditions [19]. The authors proposed that potentiation of aggregation was attributable to an increase in arachidonic acid release after 4-HNE and thrombin treatment, which would suggest an increase in levels of thromboxane A_2[19]. However, this would seem unlikely since most if not all of the exogenous HNE would be bound or react with the large amount of albumin present in PRP.

From previous studies, it is not clear if the changes seen in platelet aggregation were a direct or indirect result of HNE production. However, it is clear that 4-HNE has diverse effects on platelet aggregability, but the precise mechanisms underlying these effects are unclear. What is known is that 4-HNE reacts with nucleophilic amino acids such as cysteine, lysine, histidine, asparagine, and glutamine, and so modifies protein function [1,4,17]. Here, we used protocols to assess bioenergetics, targeted metabolomics, platelet function and 4-HNE protein adduct formation in isolated human platelets. To determine the protein targets of 4-HNE in platelets, we used an analog of HNE, A-HNE to modify and enrich for HNE-adducted proteins. A-HNE contains a terminal alkyne group which can be conjugated to a biotin tag using click chemistry allowing for affinity enrichment using neutravidin resin and identification by mass spectrometry. Our studies revealed that 4-HNE decreased thrombin-dependent aggregation and caused a depression of mitochondrial respiration which was further blunted in the presence of thrombin. On the other hand, 4-HNE caused a compensatory increase in basal glycolysis. We found that A-HNE modified an electrophilic responsive proteome comprised of 72 proteins involved in key aspects of platelet function. Taken together these data highlight the pleiotropic nature of the interaction of 4-HNE with complex biological systems and the potentially interactive targets which could result in inhibition of platelet aggregation and metabolism.

2. Methods

2.1. Platelet isolation and aggregation

For these experiments, platelets were isolated from platelet concentrates from ten donors between days 6–8 since collection. The platelet concentrates were obtained from the blood bank at the University of Alabama at Birmingham. Approval for collection and use of platelet concentrates was obtained from the University of Alabama at Birmingham Institutional Review Board (Protocol #X110718014). Platelets were isolated from the concentrates as described previously [20]. In brief, the concentrates were centrifuged at 1500 × g for 10 min and the pelleted platelets were washed with PBS containing prostaglandin I_2 (1 μg/ml) prior to determination of platelet count by turbidimetry [20-22]. Aggregation was measured by monitoring change in light transmittance at 405 nm in a 96-well plate reader, after the addition of thrombin (0.5 U/ml) [23].

2.2. Measurement of bioenergetics

Both oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured simultaneously in the extracellular flux (XF) analyzer as described previously [20,24]. In brief, platelets were pre-treated with 4-HNE (0–30 μM), alkyne HNE (30 μM) or 1-nonanol and nonanal (30 μM) for 1 h prior to performing the assay by sequential injection of oligomycin (1 μg/ml), FCCP (0.6 μM) and antimycin A (10 μM). To determine the effect of activation on bioenergetics, thrombin (0.5 U/ml) was injected prior to injection of the mitochondrial inhibitors.

2.3. Tagging platelets with A-HNE

Platelets (200×10^6) were placed in suspension in 3.6 ml XF DMEM media and incubated with 30 μM Click Tag A-HNE (4-hydroxynon-2E-nonen-8-ynal) (Cayman, Ann Arbor, MI), or unmodified 4-HNE in an equal volume of vehicle (ethanol) for 1 h at 37 °C in a non-CO_2 incubator, and Click Chemistry was performed as previously described [25]. The platelets were washed 2X with PBS and lysed in 10 mM Tris, 1% Triton X-100 containing protease inhibitor cocktail (Roche, Basel, Switzerland). The lysed samples were centrifuged at 20,000 × g for 10 min at 4 °C and the supernatant was collected and the Lowry protein assay performed. Using equal amounts of cell lysate, $NaBH_4$ (1 M) was added to the platelet lysate and incubated at room temperature (RT) for 1 h. Next, ascorbate (200 mM), cupric sulfate (100 mM) and azide-PEG3-biotin (0.5 mM, Axxora, Farmingdale, NY) were added and rotated end over end for 2 h at RT. The protein was then precipitated by adding 2X volumes of ice cold methanol, and incubating on ice for 30 min. The samples were centrifuged at 20,000 × g for 10 min at 4 °C and then the pellet was washed with ice cold methanol and re-suspended in 100 μl RIPA lysis buffer containing protease inhibitor cocktail (Roche, Basel, Switzerland). To assess the efficiency of the labeling, 10 μg of protein was subjected to SDS-PAGE gel electrophoresis and probed with streptavidin-horseradish peroxidase conjugate. For the pull down of biotinylated proteins, 75 μg of protein was incubated with 30 μl of NeutrAvidin Plus UltraLink resin (Thermo Scientific, Waltham, MA) slurry at room temperature for 1 h on a shaker. The resin was washed 6X with RIPA buffer and then 15 μl of 2X sample loading buffer containing β-mercaptoethanol was added and heated at 80 °C for 10 min. These samples were then separated on an SDS-PAGE gel and stained with Coomassie brilliant blue. To identify A-HNE modified proteins, the gel bands were excised and digested with trypsin (12.5 ng/μl) overnight, and an aliquot (5 μl) of the digest was first loaded onto a Nano cHiPLC 200 μm ID × 0.5 mm