The product of oxygenation of arachidonic acid by the prostaglandin H synthases (PGHS), prostaglandin H₂ (PGH₂), undergoes rearrangement to the highly reactive γ-ketoaldehydes, levuglandin (LG) E₂, and LGD₂. We have demonstrated previously that LGE₂ reacts with the ε-amine of lysine to form both the levuglandinlysine Schiff base and the pyrrole-derived levuglandinlysine lactam adducts. We also have reported that these levuglandinlysine adducts are formed on purified PGHSs following the oxygenation of arachidonic acid. We now present evidence that the levuglandinlysine lactam adduct is formed in human platelets upon activation with exogenous arachidonic acid or thrombin. After proteolytic digestion of the platelet proteins, and isolation of the added amino acid residues, this adduct was identified by liquid chromatography-tandem mass spectrometry. We also demonstrate that formation of these adducts is inhibited by indomethacin, a PGH synthase inhibitor, and is enhanced by an inhibitor of thromboxane synthase. These data establish that levuglandinlysine lactam adducts are formed in a PGHS-dependent pathway in whole cells, even in the presence of an enzyme that metabolizes PGH₂. They also demonstrate that a physiological stimulus is sufficient to lead to the lipid modification of proteins through the levuglandin pathway in human platelets.

Prostaglandin H synthase (PGHS) catalyzes the oxygenation of arachidonic acid to the endoperoxide, prostaglandin H₂ (PGH₂). PGH₂ is further metabolized to the prostanoids PGD₂, PGE₂, PGF₂α, thromboxane A₂, and prostacyclin by specific enzymes. Also, PGH₂ in aqueous solutions undergoes non-enzymatic rearrangement to yield PGE₂ and PGD₂, and 20% of it rearranges to the highly reactive γ-ketoaldehydes, levuglandins (LG) E₂ and D₂ (1, 2) (Fig. 1). Levuglandins are known to react covalently with primary amines, such as the ε-amine of lysine, with proteins and with DNA (3, 4). We have characterized the adducts that are formed by the reaction of lysine with LGE₂ or PGH₂ (2, 5), and knowledge of their structures has provided a basis for analysis of the adducts in protein digests utilizing liquid chromatography-tandem mass spectrometry. Utilizing this analytical approach, we have demonstrated formation of LG-lysine adducts on PGHS-1 and PGHS-2 following the oxygenation of arachidonic acid (6). Formation of covalent adducts was also observed with proteins co-incubated with PGHS and arachidonic acid. These findings formed the basis for a hypothesis that PGHS activity in cells could generate levuglandinlysine adducts of proteins.

Oxygenation of arachidonic acid by PGHS-1 in platelet microparticles has been shown to produce arachidonic acid-derived adducts of multiple proteins (7). Such labeling also has been reported in whole platelets (8) and is increased when thromboxane A₂ synthase is inhibited. However, the reactive product of arachidonic acid that forms these protein adducts has not yet been characterized. We hypothesized that these adducts of platelet proteins are formed from LGE₂ and LGD₂.

This report provides evidence that levuglandinlysine adducts of proteins are formed in cells as a consequence of the oxygenation of arachidonic acid by a PGHS.

EXPERIMENTAL PROCEDURES

Materials—Dazoxiben was a generous gift from Pfizer Ltd. (Sandwich, UK). Methanol was ordered from Burdick and Jackson (Muskegon, MI). Arachidonic acid, sodium citrate, citric acid, indomethacin, and butylated hydroxytoluene were purchased from Sigma. Sepharose 2B is from Amersham Biosciences (Uppsala, Sweden). Oasis™ Sep-Pak cartridges were obtained from Waters Corp. (Milford, MA), and dimethyl formamide and triphenylphosphine were from Aldrich. Pronase and aminopeptidase M from porcine kidney were from Calbiochem. Thrombin was obtained from Pharmingen.

Preparation of Washed Human Platelets—Human blood was obtained following a protocol approved by the Institutional Review Board of Vanderbilt University. Washed human platelets were isolated following the protocol described previously (9). The blood was drawn with a syringe containing 5 ml of 3.8% sodium citrate (final volume: 50 ml), then centrifuged in plastic tubes at 300 × g for 10 min at room temperature (23 °C). The supernatant (platelet-rich plasma) was acidified to pH 6.4 with 0.15 M citric acid (10) and then centrifuged at 1,000 g for 10 min at 4 °C. The platelet-rich plasma was washed three times with PBS (11) and pelleted at 1,000 g for 10 min at room temperature (23 °C). The supernatant (platelet-rich plasma) was acidified to pH 6.4 with 0.15 M citric acid (10) and then centrifuged at 1,000 g for 10 min at room temperature. The pellet was resuspended with 5 ml of washing buffer (24.4 mM sodium phosphate, pH 6.5, 0.113 mM NaCl, 5.5 mM glucose). After 15 min at room temperature, the platelets were purified on a Sepharose 2B column equilibrated with washing buffer. The eluted platelets were counted with a Coulter counter and diluted with resuspension buffer (8.3 mM sodium phosphate, pH 7.5, 0.109 mM NaCl, 5.5 mM glucose) for a final count of 600,000 platelets/μl.

Formation of LG-Lysine Adducts in Human Platelets—Washed platelets were then preincubated with indomethacin (final concentration of 10 μM), dazoxiben (final concentration of 10 μM), or vehicle for 30 min at room temperature. At this time, the platelets were activated by adding arachidonic acid (final concentration of 20 μM) or thrombin (final concentration of 1 U/unit/ml) and incubated at room temperature for 5 min.

Analysis of LG-Lysine Lactam Adduct in Human Platelets—After incubation, platelets were pelleted at 2,000 × g for 10 min at room temperature. After centrifugation, the LG-lysine lactam adduct was isolated from proteins and analyzed by LC MS/MS as described previously (5, 11). In short, 10 ml of cold ethanol (containing 50 mg/liter of...
butylated hydroxytoluene and 500 mg/liter of trisphenylphosphate) were added to the cell pellets, and the proteins were precipitated by centrifugation at 2,000 × g for 10 min at 4 °C. Proteins were then reprecipitated in 10 ml of cold solution of methanol/chloroform 1:2 (v/v) and washed with 10 ml of methanol (each containing butylated hydroxytoluene and trisphenylphosphate). Then, partial digestion of proteins to single amino acid was performed using 3 mg of Pronase and 3 μl of aminopeptidase M (0.15 unit) per sample. After purification the LG-lysine lactam adduct was analyzed by LC MS/MS as described previously (5, 6). The 13C-labeled internal standard was prepared by reaction of synthetic LGE2 (12) and [13C]lysine; the LG-lysine lactam standard was then purified as described previously (11).

RESULTS

We examined the formation of LG-lysine lactam adducts on platelet proteins following oxygenation of exogenous arachidonic acid. Following proteolytic digestion of platelet proteins, the products were analyzed by LC MS/MS. Selected reaction monitoring was used to analyze the LG-lysine lactam fragment ions derived from the levuglandinyl moiety (m/z 332.1) and the lysyl moiety (m/z 84.1) of the adduct (5) (Fig. 2). From platelets incubated with 20 μM arachidonic acid, both of these fragment ions are detected (Fig. 3). The simultaneous elution of the two fragment ions concurrently with the [13C]LG-lysine lactam standard provides consistent evidence that identifies the LG-lysine lactam adduct. Preincubation of the cells with indomethacin for 30 min markedly reduces the formation of the LG-lysine lactam from 169 to 20 pg of lactam/10^9 platelets. These findings demonstrate that levuglandins are generated in human platelets in a PGHS-dependent fashion and form covalent adducts with proteins.

Because of the theoretical possibility that activation of platelets with exogenous arachidonic acid might generate an amount of PGH2 that saturates its catalytic disposition by the thromboxane synthase, we also examined the formation of LG-lysine lactam adducts after activation of platelets with the physiological agonist, thrombin. As depicted in Fig. 4, LG-lysyl adducts are formed following thrombin. Inhibition of formation of the adducts by indomethacin confirms that they are derived from oxygenation of arachidonic acid by the PGHS.

Further evidence that the adducts are derived from PGH2 was obtained by examining the effect of dazoxiben, an inhibitor of thromboxane synthase. As shown in Fig. 5, inhibition of thromboxane synthase by dazoxiben led to an increase in the levels of LG-lysine lactam adduct (357 pg of lactam/1 × 10^9 platelets) by 2.1-fold compared with the control experiment in which no inhibitor was present.

DISCUSSION

This report provides the first evidence that levuglandinyl adducts of proteins can be formed in a cell as a consequence of oxygenation of arachidonic acid by a PGHS.

The immediate product of the PGHS, PGH2, is the substrate for synthases that catalyze its conversion to specific prostanoids. PGH2 also undergoes non-enzymatic rearrangement to several products, and the highly reactive ketoaldehydes, LGE2 and LGD2, account for ~20% of these products. Even though levuglandinyl adducts of proteins have been demonstrated after exposure of the proteins to PGH2 in vitro (2, 6), the competing enzymatic biotransformation of PGH2 in cells has provided a reason to question whether non-enzymatic rearrangement of PGH2 to LGE2 could occur in intact cells. To address this question, we chose to examine the platelet, in which there is robust biotransformation of PGH2 via the thromboxane synthase. As depicted in Fig. 4, the immediate product of the PGHS, PGH2, is the substrate for synthases that catalyze its conversion to specific prostanoids. Following proteolytic digestion of platelet proteins following oxygenation of exogenous arachidonic acid, we also examined the formation of LG-lysine lactam adducts on platelet proteins by the findings of Lecomte et al. (8) of uncharacterized, PGH2-derived adducts of at least 10 platelet proteins; one of these adducted proteins was PGHS-1, which we have shown to...
be adducted by levuglandin as a consequence of arachidonic acid oxygenation in vitro (6). Our finding that formation of the LG-lysine adduct of platelet proteins is inhibited by indomethacin indicates that its formation is a consequence of the oxygenation of arachidonic acid by the PGHS. The increased levels of LG-lysine adducts after treatment of the platelets with dazoxiben, on the formation of LG-lysine adducts in human platelets. Human platelets were preincubated with indomethacin 100 µM or vehicle for 30 min. After incubation with thrombin (1 unit/ml) for 15 min, the proteins from human platelets were precipitated and digested to single amino acids by step-digestion with Pronase and aminopeptidase M. The adducts were purified by chromatography on Oasis™ cartridge followed by a reverse-phase HPLC, then analyzed and aminopeptidase M. The adducts were purified by chromatography of the adducts and cross-linked proteins, they have the potential to accumulate in cells over time.

In conclusion, cyclooxygenase-dependent formation of levuglandinyl adducts of proteins occurs during platelet activation. This finding provides a basis for investigations that address the possible function of these reactive lipid adducts of proteins in platelets, as well as in other cells in which there is abundant or protracted production of PGH₂.

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