Enhancement of antinociception by coadministration of nonsteroidal anti-inflammatory drugs and soluble epoxide hydrolase inhibitors

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Combination therapies have long been used to treat inflammation while reducing side effects. The present study was designed to evaluate the therapeutic potential of combination treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) and previously undescribed soluble epoxide hydrolase inhibitors (sEHIs) in lipopolysaccharide (LPS)-challenged mice. NSAIDs inhibit cyclooxygenase (COX) enzymes and thereby decrease production of metabolites that lead to pain and inflammation. The sEHIs, such as 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester (AUDABE), stabilize anti-inflammatory epoxy-eicosatrienoic acids, which indirectly reduce the expression of COX-2 protein. Here we demonstrate that the combination therapy of NSAIDs and sEHIs produces significantly beneficial effects that are additive for alleviating pain and enhanced effects in reducing COX-2 protein expression and shifting oxylipin metabolomic profiles. When administered alone, AUDABE decreased protein expression of COX-2 to 73 ± 6% of control mice treated with LPS only without altering COX-1 expression and decreased PGE2 levels to 52 ± 8% compared with LPS-treated mice not receiving any therapeutic intervention. When AUDABE was used in combination with low doses of indomethacin, celecoxib, or rofecoxib, PGE2 concentrations dropped to 51 ± 7, 84 ± 9, and 91 ± 8%, respectively, versus LPS control, without disrupting prostacyclin and thromboxane levels. These data suggest that these drug combinations (NSAIDs and sEHIs) produce a valuable beneficial analgesic and anti-inflammatory effect while prospectively decreasing side effects such as cardiovascular toxicity.

Another approach to reduce PG formation is through suppression of COX-2 expression, which can be achieved by inhibiting NF-κB translocation with epoxycisatrienic acids (EpETEs or EETs). EETs are metabolites of arachidonic acid (AA) that undergo hydrolysis by soluble epoxide hydrolase (sEH) to generate dihydroxyeicosatrienic acids (DHETEs or DHETs). EETs decrease nuclear translocation of NF-κB (13). sEH inhibitors (sEHIs) increase the concentrations of EETs and indirectly decrease the expression of COX-2 (14).

Therefore, combinations of sEHIs and NSAIDs could be used to control inflammation and pain while reducing NSAID side effects. The purpose of the present work was to investigate the anti-inflammatory/antinociceptive effect of two sEHIs, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester (AUDABE) and 1-adamantan-1-yl-3-[5-[2-(2-ethoxyethoxy)ethoxy]pentyl]urea (AEPU) [also known as IK-950, or 1-adamantan-3-(5-(2-ethoxyethoxy)ethoxy)pentyl]urea], and three NSAIDs, rofecoxib, celecoxib, or indomethacin, by administration alone or in combination. We assessed the antinociceptive effects of these drugs by measuring thermal hindpaw withdrawal latencies in a mouse model of lipopolysaccharide (LPS)-induced inflammatory hyperalgesia (15). We hypothesized that NSAIDs would have dose-dependent additive or enhanced (more than additive) interactions with a constant dose of sEHIs.

Results

Antinociceptive Effects of NSAIDs and/or sEHIs. Nociception is the result of a complex cascade of events accomplished by diverse types of mechanisms, beginning with tissue damage in response to a noxious stimulus and subsequent release of pronociceptive and proinflammatory mediators. Alteration of these proinflammatory mediators is a current therapeutic approach to alleviate pain and inflammation. Here we compare the sEHIs with current drugs, including indomethacin, celecoxib, and rofecoxib. Mice (C57BL/6) injected with LPS (10 mg/kg; i.p.) develop hyperalgesia, indicated by a decrease in paw withdrawal latency to a noxious thermal stimulus. Upon LPS challenge, the latency for hindpaw withdrawal drops to 46 ± 7% as compared with mice administered saline vehicle. Prophylactic administration of rofecoxib (10 or 25 mg/kg), celecoxib (25, 50, or 100 mg/kg), or indomethacin (25, 50, or 100 mg/kg) produces a dose-dependent...
antihyperalgesic effect (Fig. 1). The sEHI, AUDA-BE (20 mg/kg), also elicits an antihyperalgesic effect equivalent to that of the low dose of rofecoxib (10 mg/kg; Fig. 2a). Coadministration of AUDA-BE (20 mg/kg) with low dose of rofecoxib (10 mg/kg), celecoxib (50 mg/kg), or indomethacin (50 mg/kg) has an additive effect in reducing thermal hyperalgesia (Fig. 2a). An additional sEHI, AEPU, that is structurally different, was also evaluated for its ability to reduce hyperalgesia and was used in combination with the lower doses of rofecoxib (10 mg/kg), celecoxib (50 mg/kg), or indomethacin (50 mg/kg) (see Fig. 2b for structures). Although similar trends were observed with this more polar sEHI, the results in the hindpaw withdrawal assay
and oxylipin metabolite profiles were more variable, possibly due to the compound’s pharmacokinetic parameters (see Fig. 7, which is published as supporting information on the PNAS web site).

Effects of NSAIDs or sEHIIs on PG Synthesis. As expected, the NSAIDs reduced production of PGD$_2$ and PGE$_2$ in a dose-dependent manner. Previous work has shown that AUDA-BE indirectly reduced PGD$_2$ and PGE$_2$ in a dose-dependent manner (14). More specifically, a dose of AUDA-BE at 20 mg/kg reduces the levels of PGD$_2$ and PGE$_2$ by 31 ± 9% and 34 ± 6% compared with LPS, respectively, which is approximately equivalent to rofecoxib’s antinociceptive efficacy at a dose of 10 mg/kg (Fig. 3).

When AUDA-BE (20 mg/kg) is administered in combination with low doses of NSAIDs, there is an additive or enhanced effect in reducing PGD$_2$ and PGE$_2$ concentrations. Specifically, coadministration of indomethacin (25 mg/kg) and AUDA-BE (20 mg/kg) reduces the PGD$_2$ by 68 ± 6% and PGE$_2$ by 51 ± 7%, respectively, which is comparable with rofecoxib’s antinociceptive efficacy at a dose of 10 mg/kg (Fig. 3).

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COX-2 protein in vivo. This effect may be caused by COX inhibitors diverting AA from the COX to the P450 metabolic pathway, thus increasing the concentration of EETs.

High concentrations of PGD2 and PGE2 alone can induce COX-2. Here we hypothesize that AUDA-BE decreases the amount of transcribed COX-2 and that celecoxib directly inhibits the enzyme producing less PGD2 and PGE2, thus depressing the positive feedback loop. In addition, the coadministration elevates the concentration of the EETs, which would further decrease COX-2 expression (Fig. 5; see also Fig. 9, which is published as supporting information on the PNAS web site).

Effects of NSAIDs or sEHIs on Epoxide Synthesis. Approximately one-third of the AA carbon flow is metabolized by the COX enzymes, which is disrupted by administration of NSAIDs. This disruption shifts AA metabolism to the P450 and sEH or the lipoxygenase pathway and inevitably produces more EETs and DHETs or hydroxyeicosatetraenoic acids (HETEs). Fig. 5 depicts the shift in metabolism through the P450 and sEH pathways, as well as the effects of coadministration of AUDA-BE. In all cases the combination therapy with high doses of the NSAIDs doubled the concentration of EETs in the plasma after LPS challenge.

Potential Decrease in Side Effects. Given that selective COX-2 inhibitors such as rofecoxib and celecoxib block the formation of vascular endothelial cell prostacyclin (PGI2, stable metabolite 6-keto-PGF1α), but not platelet COX-1 derived thromboxane A2 (TXA2) (stable metabolite TXB2) the large decrease in the PGI2-to-TXA2 ratio with COX-2 inhibitors may account for the increased incidence of thrombotic cardiovascular events. In contrast, when AUDA-BE is administered alone or in combination with COX-2 inhibitors, the relative ratio of PGI2 (6-keto-PGF1α) to TXA2 (TXB2) in plasma is not significantly altered from the ratio in LPS-challenged mice (Fig. 6; see also Fig. 10, which is published as supporting information on the PNAS web site). The data indicate that a combination therapy of sEHIs with low doses of rofecoxib or celecoxib results in the desired decrease in inflammatory eicosanoids like PGD2 and PGE2 (Figs. 3 and 8) without the changes in 6-keto-PGF1α-to-TXB2 ratio associated with undesirable cardiovascular risks. The mechanism by which 6-keto-PGF1α is elevated remains unknown. A potential hypothesis is that PG-I synthase, which produces PGI2, is up-regulated by the sEHIs or EETs. Certainly, if some NSAID side effects are compound related, a sEHI–NSAID combination permits a reduced NSAID dose and concomitant risk.

Conclusions

The goal of analgesic drug combinations is to optimize dose regimes that offer greater analgesic and anti-inflammatory...
effects, while at the same time decreasing detrimental side effects. Recently, much attention has focused on the potential of COX-2 inhibitors to increase myocardial infarction and stroke risk (17) and delay resolution of inflammation (18). Our previous work has shown that AUDA-BE has little effect on 6-keto-PGF$_\alpha$ and TXB$_2$ imbalance associated with increased risk for thrombotic events and appears to promote the formation of proresolution anti-inflammatory mediators. Therefore, the combination of conventional/established NSAIDs and sEHIs appears to be a useful approach for decreasing pain and inflammation while avoiding unwanted side effects.

The dose of AUDA-BE (20 mg/kg) plus indomethacin (25 or 50 mg/kg) had an additive effect in reducing PGD$_2$/PG$_E$ synthesis and hyperalgesia, respectively. The dose of AUDA-BE (20 mg/kg) plus rofecoxib (10 mg/kg) or celecoxib (25 mg/kg) had an enhanced effect in reducing PGD$_2$ and PG$_E$ synthesis and an additive effect in reducing hyperalgesia without negatively impacting the 6-keto-PGF$_\alpha$ concentration in plasma.

The full mechanism underlying the interaction between the NSAIDs and sEHIs remains unknown. Inflammation and pain are controlled not only by oxypins but also by multiple mediators including K$^+$, ATP, substance P, bradykinin, cytokines, monoaoms, lipid amides, and steroids. Because there are multiple inflammatory mediators that sensitize/activate nociceptors other than those affected by COX/sEH inhibition, it is not surprising that a combination therapy has only an additive effect in this model, while demonstrating enhanced effects where the two classes of inhibitors intersect. However, it is feasible that the mechanism of inhibiting COX-2 induction through sEHIs and then directly inhibiting the enzyme with NSAIDs would reduce proinflammatory mediators and pain. Alternatively, the mechanism could be due to different actions and sites of action of the inhibitor groups.

In summary, systemic coadministration of low-dose NSAIDs and sEHIs has the following effects: (i) produces at least an additive antinociceptive effect in an inflammatory pain model; (ii) produces an enhanced effect in decreasing PGD$_2$ and PG$_E$ levels that is greater than the sum of individual treatments; and (iii) does not result in the imbalance in 6-keto-PGF$_\alpha$ and TXB$_2$ usually associated with COX-2 inhibitors. The data indicate that sEHIs and EETs can enhance the effects of NSAIDs and allow reduced doses of NSAIDs to be used for the same therapeutic effect. Similarly, low doses of aspirin should synergize the effect of sEHIs. These combinations should lead to more indomethacin-like than rofecoxib-like ratios of PGF$_2$ to TXA$_2$. These results demonstrate that sEHIs may have utility when combined with low doses of conventional NSAIDs as useful agents for the treatment of pain and inflammation. This observation may extend to other physiological processes associated with increased/decreased concentration of epoxy fatty acids.

Materials and Methods
Behavioral Nociceptive Tests and Treatments. Behavioral nociceptive testing was conducted by assessing thermal hindpaw withdrawal latencies using a commercial Hargreaves apparatus (IITC, Woodland Hills, CA) according to procedures described by Woolfe and McDonald (19). Briefly, mice were placed in an acrylic experimental chamber with a glass surface. The temperature of this surface was maintained at 30°C. Before data collection, mice were acclimated to the experimental chambers in 30-min sessions daily for 3 days. On the 4th day, baseline readings were taken. After a 30-min period of acclimation, noxious radiant heat was applied to the plantar surface of the hindpaw. Five measurements per animal were taken in 2-min intervals, and these five values were averaged at each pre- and post-LPS measurement. The latency to withdraw the paw away from the thermal stimulus was recorded (seconds). Trioleate or 0.5% carboxymethylcellulose was then injected s.c. as vehicle controls for therapeutic agents in LPS-treated animals. Saline (0.9%) was injected i.p. as a vehicle control for the LPS injection. Measurements were taken 6 h later. Two days later, the same animals were s.c. injected with either NSAIDs or sEHIs, alone and in combination; 24 h later, they were i.p. challenged with LPS, immediately followed by another dose of the therapeutics. Six hours after LPS challenge, hindpaw withdrawal latency tests were conducted, and animals were killed for blood and tissue sampling. This dosing and sampling schedule was chosen based on previous work to obtain therapeutic levels as well as clear changes in hepatic COX-2 protein levels.

Dose Response. The antinociceptive effects produced by rofecoxib, celecoxib, indomethacin, AUDA-BE, and AEPUs were evaluated individually and in combination. First, each dose of rofecoxib [(COX2 IC$_{50}$, 18.0 nM) 10 and 25 mg/kg], celecoxib [(COX2 IC$_{50}$, 40.0 nM) 25, 50 and 100 mg/kg], or indomethacin [(COX1: COX2 IC$_{50}$, 0.028: 1.68 μM) 25, 50 and 100 mg/kg], was given to four animals to obtain the corresponding dose–response. The doses were chosen in the therapeutic range from preliminary pharmacokinetic data, eicosanoid profiles, and current literature (20–23). For instance, celecoxib was administered at higher doses because it has a larger volume of distribution and is less efficacious when compared with rofecoxib.

The antinociceptive effects of AUDA-BE and AEPUs were individually tested at 20 mg/kg. Previous work has indicated that 20 mg/kg is an effective dose at reducing acute inflammation (15). AUDA-BE and AEPUs have quite different structures and physical properties. For example, their experimental water solubilities (5 and 120 μg/ml) and calculated logP values (4.19 and 1.86, respectively) are quite different. The fact that both sEHIs give similar results supports the hypothesis that enzyme inhibition is the common mechanism of action; however, they could interact with targets other than sEH. For example, as anticipated from the structure, AUDA-BE acts as a weak peroxisome proliferator-activated receptor (PPAR) α-agonist, whereas AEPUs is unlikely to be an agonist. Both sEHIs have low-nanomolar IC$_{50}$ values with AUDA-BE being slightly more potent. However, the higher efficacy of AUDA-BE in these studies is because of its longer in vivo half-life and volume of distribution (see Fig. 7).

Then the doses of rofecoxib, celecoxib, and indomethacin were each combined with a fixed dose of AUDA-BE and AEPUs to analyze possible interactions. Mice were prophylactically administered s.c. the inhibitors 24 h before LPS (10 mg/kg) i.p. challenge. Immediately after the LPS exposure, another dose of the inhibitors was administered s.c. Trioleate and 0.5% carboxymethylcellulose were administered at the corresponding volume as a vehicle control in LPS-treated animals. At the end of the experiment, 6 h after the LPS challenge, the mice were killed, and blood, livers, spleens, and kidneys were collected for biochemical and Western analysis. Blood was collected by cardiac puncture with an EDTA-rinsed syringe. A combination of triphenylphosphine, butylated hydroxytoluene, and indomethacin (0.2% for each, w/t/wt) was added to each collection tube. The samples were immediately spun; the plasma was separated and flash frozen in liquid nitrogen. All samples were stored at −80°C until analysis.

Data Analysis. Results are expressed as average ± SD and are depicted as percentages of controls mice receiving vehicle without LPS. Statistical comparisons were analyzed by Student’s t test and one-way ANOVA followed by Tukey or Dunnnett’s test for post hoc comparison. Statistical significance was considered to be achieved when P < 0.05.

Statistical significance between the theoretical additive and experimentally derived values were evaluated with Student’s t test. An experimental value that was significantly lower than the
theoretical additive value was considered to indicate an enhanced interaction between sEH inhibition and NSAID (24). To determine additive or enhanced effect, a theoretical decrease in response (e.g., PGE2) was calculated by addition of percent decrease of the individual inhibitors. For example, indomethacin (10 mg/kg) decreased PGE2 by 11% as compared with LPS without treatment, and AUDA-BE decreased by 40%; therefore, the theoretical additive effect would be 51%. This theoretical value was then compared with the actual decrease found for coadministration of these inhibitors at the specified doses. If the actual value was greater than the theoretical value, then the combination therapy was considered to be enhanced.

For further details, see Supporting Materials and Methods, which is published as supporting information on the PNAS website.

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