Adenosine, a Potent Natural Suppressor of Arachidonic Acid Release and Leukotriene Biosynthesis in Human Neutrophils

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Leukotrienes (LTs) constitute a family of arachidonic acid (AA) metabolites with potent biological activities. Leukotriene B_4 and the cysteinyl-LTs (LTC_4, -D_4, and -E_4) have now been implicated in a number of physiological processes and inflammatory and allergic diseases (1). The early demonstration of an involvement of the cysteinyl-LTs as mediators of the bronchospasm in asthma has provided the impetus for the development of LT synthesis inhibitors and LT antagonists, and stimulated intensive research toward the understanding of the mechanisms of LT synthesis. While our knowledge of the biochemistry of LT synthesis and the enzymes involved has made remarkable progress, particularly with the identification and cloning of the proteins of the 5-lipoxygenase (5-LO) pathway, much remains to be learned about the complex mechanisms of regulation of the biosynthesis of these important mediators of inflammation and allergy.

STIMULATION OF LEUKOTRIENE BIOSYNTHESIS IN NEUTROPHILS

LT synthesis in the peripheral blood neutrophil is primarily dependent on AA release and activation of 5-LO (2–4); indeed, the concentration of free AA is kept low in cells by the constant reacylation of the fatty acid released in deacylation processes. Therefore as with other eicosanoids, LT synthesis is limited by substrate availability and depends on the stimulation of the release of AA (mainly from phospholipids).

Since the discovery of the 5-LO pathway, pharmacological agents such as the divalent cation ionophores A23187 and ionomycin have been widely used to trigger the synthesis of LT in cells, tissues, and organs. The ionophores, the most powerful stimuli of LT synthesis so far described, likely act by causing a long-lasting elevation of intracellular Ca^{2+} concentration, which induces massive translocation of both 5-LO and cytosolic phospholipase A_2 ($cPLA_2$) from the cytosol to the nuclear envelope (5, 6). In sharp contrast, the natural neutrophil agonists such as the lipidic and peptidic chemoattractants (platelet-activating factor [PAF], formyl-methionyl-leucyl-phenylalanine [fMLP], complement component C5a, and interleukin 8 [IL-8]) induce only minimal LT synthesis (one to three orders of magnitude inferior to that induced by optimal concentrations of A23187), indicating that circulating neutrophils demonstrate a low reactivity (in terms of LT synthesis) to its soluble agonists (3, 4, 7). Studies implicating additions of exogenous substrate to neutrophil suspensions stimulated with agonists have clearly established that 5-LO was activated by the agonists and that failure of the cells to generate LTs could be largely attributed to insufficient release of AA (4).

Numerous studies have documented the "priming" effects of a number of hematopoietic growth factors (mainly granulocyte-macrophage colony-stimulating factor [GM-CSF]), cytokines (mainly tumor necrosis factor α [TNF-α]), and bacterial products such as lipopolysaccharides (LPS) on phagocytes. Granulocytes exposed to priming agents show enhanced functional responses (degranulation, phagocytosis, superoxide anion production) to a second stimulus such as chemoattractants, and also show increased capacity for the synthesis of LTs (8–11).

Therefore, while circulating neutrophils show minimal response (LT synthesis) to their natural agonists, exposure to proinflammatory agents such as GM-CSF and TNF-α, which are present in inflammatory exudates, results in a significant enhancement of the capacity of neutrophils to produce 5-LO products. The mechanism of priming was shown to involve both cellular events required for LT synthesis, the activation of 5-LO (12), and the release of substrate (9, 11, 13). The priming of emigrated neutrophils by growth factors, cytokines, and LPS likely represents a crucial event in the regulation of LT synthesis and other phagocyte functions, allowing these cells to efficiently execute their role in host defense. The release of lipidic inflammatory mediators into the extravascular space may contribute to promote (through cell recruitment) or modulate the inflammatory process.

INHIBITION OF LEUKOTRIENE BIOSYNTHESIS IN HUMAN NEUTROPHILS

The preceding sections emphasize that much effort has been put into understanding the mechanisms by which LT biosynthesis is upregulated in human neutrophils. In contrast, little is known of the putative mechanisms of suppression of LT synthesis. A limited number of studies, however, clearly indicate that such downregulation mechanisms do exist. Indeed, already in 1983, Ham and colleagues (14) demonstrated that E-type prostaglandins were potent inhibitors of LT synthesis induced by the chemoattractant fMLP in human neutrophils. Ten years later, Fonteh and colleagues (15) demonstrated that the β-adrenergic receptor agonist isoproterenol, as well as phosphodiesterase inhibitors, blocked ionophore A23187-induced LT synthesis and AA release in human neutrophils, and suggested an implication of elevated cellular levels of cyclic AMP in mediating these inhibitory effects. Such studies aimed at the identification and characterization of natural mechanisms of suppression of LT synthesis are most important since the physiological regulation of LT synthesis likely involves a balance between stimulatory

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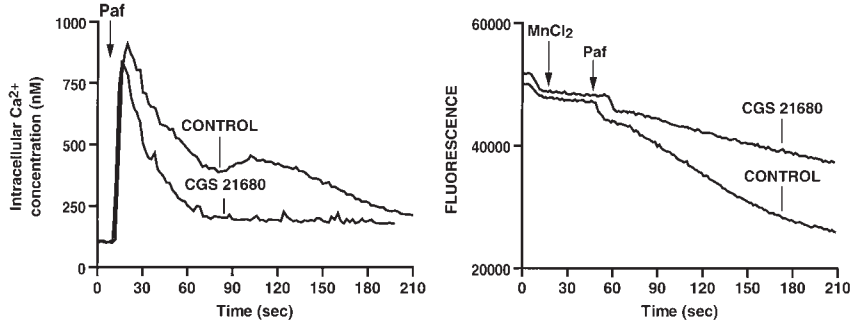

Figure 1. Effect of the A_{2a} receptor agonist CGS-21680 on PAF-induced changes in $[Ca^{2+}]_i$ and Mn^{2+} influx. *Left*: Fura-2-loaded neutrophils (5×10^6/ml) were pretreated with ADA (0.1 U/ml) for 5 min in the absence (control) or the presence of 1 μM CGS-21680 at 37° C. The cells were then stimulated with 300 nM PAF and the fluorescence was monitored at the excitation and emission wavelengths of 340 and 510 nm, respectively. *Right*: Fura-2-loaded neutrophils (5×10^6/ml) were washed once and resuspended in Ca^{2+}-free HBSS and pretreated with ADA (0.1 U/ml) for 5 min in the absence (control) or presence of 1 μM CGS-21680 at 37° C; Mn^{2+} (final concentration, 100 μM) was added and the cells were stimulated with 300 nM PAF. The quenching of intracellular Fura-2 fluorescence by Mn^{2+} was monitored at the excitation and emission wavelengths of 360 and 510 nm, respectively. Results are from one experiment representative of at least three others. $[Ca^{2+}]_i$ = intracellular Ca^{2+} concentration.

and inhibitory mechanisms; such a balance of positive and negative regulatory mechanisms may be altered in disease states, including asthma and other inflammatory diseases. Considering the demonstrated implication of LTs in inflammatory and allergic diseases, it seems essential to achieve a comprehensive understanding of both stimulatory and inhibitory mechanisms of LT synthesis, which may result in the discovery of novel therapeutic approaches to inflammatory diseases.

In this regard, the following sections summarize our studies demonstrating that adenosine, acting through inhibition of agonist-induced AA release, is a potent natural suppressor of LT synthesis in human neutrophils.

ADENOSINE SUPPRESSES NEUTROPHIL FUNCTIONS

Adenosine is a ubiquitous autacoid with a large spectrum of biological activities, including the modulation of leukocyte functions. Indeed, adenosine, via occupancy of A_{2a} receptors on neutrophils, inhibits their adherence to endothelial cells, the generation of superoxide anions, and phagocytosis (16). Moreover, adenosine was shown to inhibit the synthesis of proinflammatory cytokines by LPS-treated monocytes (17, 18) and macrophages (19). In lymphocytes, adenosine inhibits the synthesis of immunoglobulins (20) and lymphocyte-mediated cytolysis (21). *In vivo* studies have demonstrated a protective role of adenosine and its structural analogs in models of acute inflammation such as experimental adjuvant arthritis (22), ischemia–reperfusion (23–26), and carrageenan-induced pleural inflammation (27). Furthermore, methotrexate, an antifolate commonly used in the treatment of patients with rheumatoid arthritis, causes accumulation of adenosine and inhibition of leukocyte migration in inflammatory exudates in mice (28). For these reasons, adenosine is increasingly viewed as a potent antiinflammatory agent.

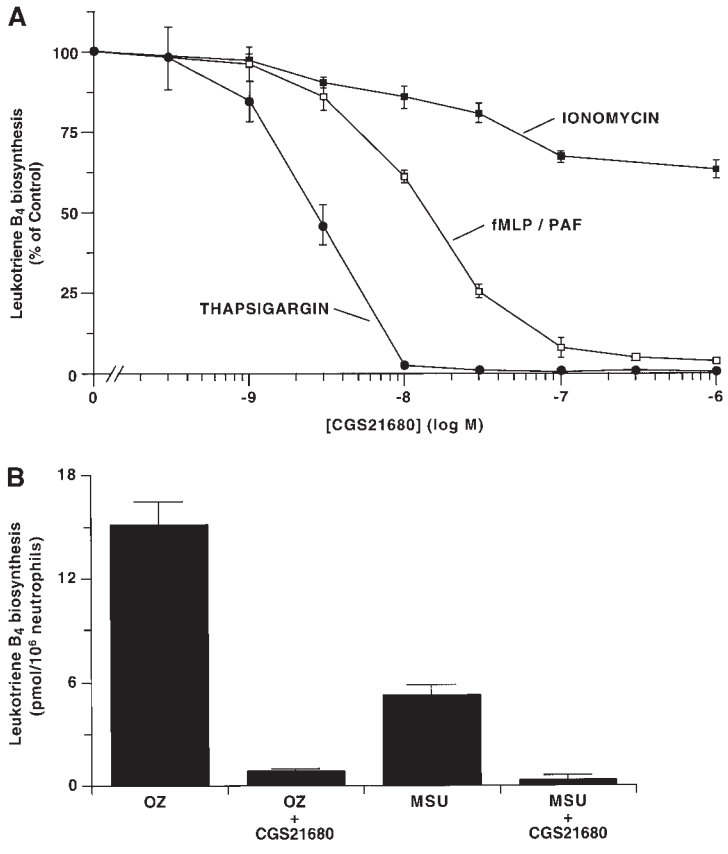

Figure 2. Effect of the A_{2a} receptor agonist CGS-21680 on the biosynthesis of LTB_4 in human neutrophils. (*A*) Neutrophils (5×10^6/ml) were pretreated with 700 pM GM-CSF plus 1.2 nM TNF-α for 30 min at 37° C and then treated for 5 min with adenosine deaminase (0.1 U/ml) and increasing concentrations of CGS-21680 before stimulation with either ionomycin (1 μM), the neutrophil agonist fMLP and PAF (each at 300 nM at 5 min intervals), or thapsigargin (100 nM) and further incubated for 5 min (ionomycin), 10 min (thapsigargin), or 10 min (fMLP/PAF) at 37° C. Control levels of LTB_4 biosynthesis (100%) were 230 ± 15, 9 ± 2, and 220 ± 20 ng in neutrophil suspensions stimulated with ionomycin, fMLP/PAF, and thapsigargin, respectively. (*B*) Neutrophils (5×10^6/ml) were pretreated with GM-CSF and TNF-α as described above and then treated for 5 min with adenosine deaminase (0.1 U/ml) in the presence or absence of CGS-21680 (1 μM) before stimulation with opsonized zymosan (OZ) or monosodium urate crystal (MSU) (1 mg/ml), further incubated for 20 min (OZ) or 30 min (MSU) at 37° C. LTB_4 biosynthesis was measured by reversed-phase HPLC as described previously (38); amounts of LTB_4 indicated represent the sum of LTB_4, 20-OH-LTB_4, and 20-COOH-LTB_4. The data are the mean (± SD) of triplicate incubations from one of three representative experiments.

The mechanism by which adenosine inhibits neutrophil functions is not fully understood; however, it is clear that these inhibitory effects of adenosine involve the adenosine A_{2a} receptor and that engagement of this receptor results in increased levels of intracellular cyclic AMP (29). Elevation of intracellular cyclic AMP levels has long been recognized as a cellular event leading to inhibition of cell functional responses in neutrophils. Other hypotheses for the inhibitory effect of adenosine on neutrophil functions include uncoupling of chemoattractant receptors from their signal transduction mechanisms (30), or the stimulation of a serine/threonine phosphatase (31). Another consequence of adenosine stimulation of human neutrophils is the inhibition of agonist-induced Ca^{2+} influx (32) (Figure 1). Given the crucial role of Ca^{2+} in most functional responses of neutrophils and in particular in the activation of enzymes required for LT synthesis, i.e., $cPLA_2$ and 5-LO (33, 34), it was tempting to speculate that the autacoid may also exert a suppressive effect on neutrophil LT synthesis. Studies were thus undertaken to investigate the effects of adenosine on the synthesis of proinflammatory lipid mediators.

ADENOSINE INHIBITS LEUKOTRIENE BIOSYNTHESIS IN NEUTROPHILS

To avoid possible difficulties of interpretation of data resulting from the interaction of adenosine with several adenosine receptor subtypes present on leukocytes (35), studies aimed at investigating the putative inhibitory effect of adenosine on LT biosynthesis were performed with an analog of adenosine demonstrating high selectivity for the A_{2a} adenosine receptor, CGS-21680 [2-*p*-(2-carboxyethyl)phenetylamino-5′-*N*-ethylcarboxamidoadenosine HCl] (35). Our studies demonstrated that CGS-21680 (and other adenosine analogs) were potent inhibitors of LTB_4 biosynthesis, both in heparinized whole blood stimulated with fMLP and in isolated neutrophils also stimulated with agonists (Figure 2A) (36). Studies with analogs of adenosine selective for the A1 or A_{2a} receptor, as well as with selective A_{2a} receptor antagonists, clearly established that the inhibitory effect of adenosine on LT biosynthesis involves engagement of the A_{2a} receptor on neutrophils (36). Interestingly, other studies clearly indicated that adenosine was not equally potent in inhibiting eicosanoid biosynthesis in various cell types; heparinized whole blood and isolated neutrophils were most sensitive to inhibition of LTB_4 synthesis by CGS-21680 (36). Blood monocytes (36) and alveolar macrophages were much less sensitive, with a median inhibitory concentration (IC_{50}) of ~ 1 μM, and eosinophils and platelets were even more resistant to the inhibitory effect of CGS-21680 with IC_{50} values above 1 μM [Table 1 (37–39)]. The reason for these marked differences in the sensitivity of various cell types to eicosanoid biosynthesis inhibition by CGS-21680 is not clear; it may be explained by differences in the level and/or function of the A_{2a} receptors between these various cell types (40, 41).

The ability of CGS-21680 to inhibit LTB_4 synthesis in neutrophils also varied depending on the stimulus used to activate LT biosynthesis; Figure 2A shows that while CGS-21680 is a weak inhibitor of ionomycin-induced LTB_4 biosynthesis, it potently inhibits LTB_4 biosynthesis induced by agonists in neutrophils primed with GM-CSF and TNF-α. Surprisingly, CGS-21680 was an even more potent inhibitor of thapsigargin-induced LTB_4 biosynthesis in neutrophils. The ability of the A_{2a} receptor agonist to inhibit LTB_4 biosynthesis induced by opsonized phagocytic particles was equivalent to that shown with the neutrophil soluble agonists (Figure 2B and Table 2) and inhibition of the ionophore A23187-induced biosynthesis was similar to ionomycin, showing an IC_{50} above 1 μM (Table 2 and Reference 36). The ability of CGS-21680 to inhibit thapsigargin-induced LTB_4 biosynthesis as opposed to A23187- or ionomycin-induced biosynthesis is somewhat intriguing; it must be emphasized, however, that A23187 and ionomycin act as ionophores, causing an intense and sustained accumulation of Ca^{2+} in the cells, whereas thapsigargin has a different mechanisms of action, acting as an inhibitor of the endomembrane Ca^{2+} ATPase and causing a release of Ca^{2+} from intracellular stores that results in a massive influx of extracellular Ca^{2+} through Ca^{2+} channels (42).

ADENOSINE INHIBITS ARACHIDONIC ACID RELEASE IN ACTIVATED NEUTROPHILS

We next addressed the question of the mechanism by which A_{2a} receptor engagement results in inhibition of LTB_4 synthesis in neutrophils. Because it is recognized that the availability of substrate, i.e., AA, is a limiting factor in the biosynthesis of eicosanoids in cells and tissues, and because it has already been observed in several cells (including neutrophils) producing LTs that elevation of intracellular cyclic AMP levels re-

TABLE 1

SENSITIVITY OF VARIOUS HUMAN CELLS AND BLOOD TO INHIBITION OF EICOSANOID BIOSYNTHESIS BY THE A_{2a} RECEPTOR AGONIST CGS-21680*

Cell Types	IC_{50} (CGS-21680)
Whole blood	~ 10 nM
Neutrophils	~ 10 nM
Monocytes	~ 1 μM
Alveolar macrophages	~ 1 μM
Eosinophils	> 1 μM
Platelets	> 1 μM

* Heparanized whole blood, isolated neutrophils, and monocytes were pretreated with LPS and TNF-α and stimulated with 1 μM fMLP as described previously (37); human alveolar macrophages obtained by bronchoalveolar lavages were directly stimulated with LPS (1 μg/ml) or fMLP (1 μM); human eosinophils were isolated from the blood of patients with mild asthma using anti-CD16 magnetic beads, pretreated with GM-CSF for 90 min at 37° C, and then stimulated with PAF (1 μM) for 10 min and with C5a (10 nM) for a further 10 min at 37° C. Platelet-rich plasma was obtained from EDTA–blood and isolated platelets were stimulated with thrombin (1 U/ml) for 15 min at 37° C. All incubations were carried out in the absence or presence of increasing concentrations of CGS-21680 and in the presence of adenosine deaminase (0.1 U/ml) (except in blood) added 5 min before stimulation of eicosanoid biosynthesis. The generation of eicosanoids was measured by reversed-phase HPLC as described previously (38, 39); the IC_{50} values shown are for the inhibition of LTB_4 biosynthesis (whole blood, neutrophils, monocytes, and alveolar macrophages), LTC_4 biosynthesis (eosinophils), and 12-hydroxyeicosatetraenoic acid and 12-hydroxyheptadecatrienoic acid (platelets).

TABLE 2

IC_{50} VALUES FOR THE INHIBITION OF LTB_4 BIOSYNTHESIS IN HUMAN NEUTROPHILS INDUCED BY VARIOUS AGENTS*

Agents	IC_{50} (CGS-21680)
Thapsigargin	1–10 nM
Soluble agonists	~ 10 nM
Phagocytic particles	~ 10 nM
A23187	> 1 μM
Ionomycin	> 1 μM

* Human neutrophils (5×10^6/ml) were stimulated with either thapsigargin (30 nM to 1 μM), fMLP or PAF (1 μM), opsonized zymosan particles (1 mg/ml), the ionophore A23187 (10 nM to 1 μM), or ionomycin (30 nM to 1 μM), in the presence or absence of increasing concentrations of CGS21680 and in the presence of adenosine deaminase (0.1 U/ml) added 5 min before LTB_4 biosynthesis stimulation. Neutrophil suspensions stimulated with soluble agonists or phagocytic particles were previously primed with GM-CSF and TNF-α, or with LPS and TNF-α (as described previously). LTB_4 biosynthesis was measured by reversed-phase HPLC (38, 39).

sults in inhibition of AA release (15), we investigated the effect of the neutrophil A_{2a} receptor engagement on AA release in LPS- and TNF-α-primed neutrophils stimulated with the soluble agonist PAF. Figure 3 clearly shows that the rapid rise in the release of AA by neutrophils activated with PAF was almost completely abolished by treatment with CGS-21680. CGS-21680 was also shown to completely inhibit AA release in thapsigargin-activated neutrophils (data not shown). Because the release of AA in activated neutrophils is a Ca^{2+}-dependent process (43), it was tempting to speculate that the previously reported inhibitory effect of adenosine on agonist-induced Ca^{2+} influx in neutrophils [(32); *see also* Figure 1] might account for the inhibition of AA release observed in these experiments. However, a simple experiment measuring the ability of human neutrophils to produce LTB_4 in the presence or absence of extracellular Ca^{2+} immediately ruled out this hypothesis. Indeed, the human neutrophils activated with PAF in the absence of extracellular Ca^{2+} (cells incubated in Ca^{2+}-free Hanks' balanced salt solution [HBSS] or in HBSS containing Ca^{2+} plus 2 mM EGTA) consistently produced two to three times more 5-LO products compared with cells incubated in normal HBSS (containing 1.6 mM Ca^{2+}); furthermore, this enhanced LT biosynthesis in absence of extracellular Ca^{2+} was inhibitable by CGS-21680 (Figure 4).

It is noteworthy that under experimental conditions in which CGS-21680 is found to profoundly inhibit AA release, the secreted PLA_2 ($sPLA_2$) inhibitor SB-203347 had no inhibitory effect on AA release at concentrations up to 10 μM, whereas the $cPLA_2$ inhibitor MAFP (methylarachidonyl-fluorophosphonate) inhibited AA release by more than 50% at a concentration of 10 nM and by 80% at a concentration of 1 μM, strongly supporting the involvement of $cPLA_2$ in LPS-primed and agonist-stimulated human neutrophils (44). The activation of $cPLA_2$ is believed to involve (at least) two separate events: $cPLA_2$, which is a cytosolic enzyme in resting neutrophils, undergoes a Ca^{2+}-dependent translocation to nuclear structures on cell activation; in addition, $cPLA_2$ undergoes phosphorylation of Ser-505, which results in an increase in its catalytic activity (43, 45). This specific phosphorylation event of $cPLA_2$ is detectable by Western blot analysis since phosphorylation of Ser-505 results in decreased electrophoretic mobility (band shift). We investigated the consequence of neutrophil A_{2a} receptor engagement on these two events (phosphorylation and translocation) linked to the stimulation of $cPLA_2$ activity. In several experiments in which CGS-21680 completely inhibited the biosynthesis of LT in LPS-primed and PAF-stimulated neutrophils, there was no measurable effect of the adenosine analog on $cPLA_2$ translocation to neutrophil nuclei, or on its phosphorylation (Ser-505), as observed by band shift on Western blot analysis (data not shown). These data suggest that engagement of the A_{2a} receptor causes an inhibition of AA release in activated neutrophils through an as yet unknown mechanism, distinct from the mechanisms previously described for the activation of $cPLA_2$. Hypothetically, A_{2a} receptor engagement may interfere with the transfer of AA between classes of phospholipids, a process involving a CoA-independent transacylase (46) and shown to be required for LT biosynthesis to occur. Alternatively, the A_{2a} agonist may induce specific phosphorylation events on $cPLA_2$ (distinct from Ser-505 phosphorylation) that may result in the inhibition of the catalytic activity of $cPLA_2$; in this regard, it has been shown that $cPLA_2$ can be phosphorylated at sites other than Ser-505; the functional consequences of these phosphorylation events are, however, yet unknown (47).

ADENOSINE INHIBITS LTB_4 BIOSYNTHESIS VIA CYCLIC AMP

The neutrophil A_{2a} receptor is coupled to adenylate cyclase and its engagement results in enhanced cyclic AMP levels in neutrophils (29); in addition, previous studies have shown that several agents that cause an elevation of intracellular levels of cyclic AMP in neutrophils, namely PGE_2, the β-adrenergic agonist isoproterenol, and the type IV phosphodiesterase inhibitor rolipram, all inhibit LT biosynthesis and/or AA release in acti-

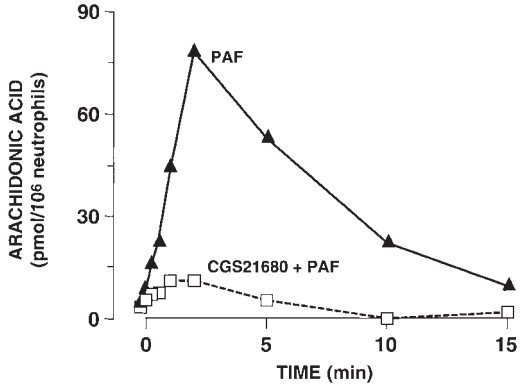

Figure 3. Effect of the A_{2a} receptor agonist CGS-21680 on AA release in activated neutrophils. Human neutrophils (5×10^6/ml) were pretreated with LPS and TNF-α for 30 min at 37° C as described in the caption to Figure 2; the cell suspensions were then treated with adenosine deaminase (0.1 U/ml) and simultaneously exposed or not exposed to 1 μM CGS-21680 5 min before stimulation with 300 nM PAF for the indicated times. AA was isolated from incubation medium by reversed-phase HPLC and analyzed by liquid chromatography-mass spectrometry (electrospray ionization) as described previously (13). The data shown are from single incubations, from one experiment representative of three others.

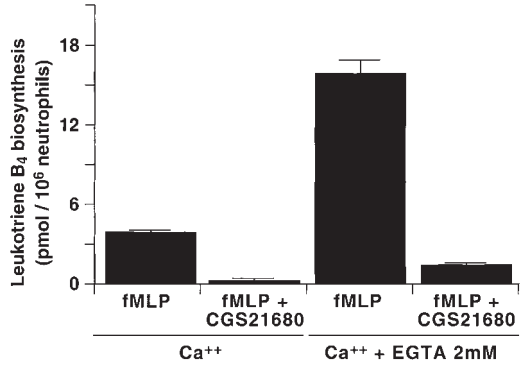

Figure 4. Effect of extracellular Ca^{2+} removal on LTB_4 biosynthesis in activated human neutrophils. Neutrophils (5×10^6/ml) in suspension in HBSS containing 1.6 mM $CaCl_2$ were pretreated with GM-CSF and TNF-α for 30 min at 37° C as described in the caption to Figure 2; the cell suspensions were then treated for an additional 5 min with adenosine deaminase (0.1 U/ml) and simultaneously exposed or not exposed to 1 μM CGS-21680. EGTA was added (or not) to the neutrophil suspensions to a final concentration of 2 mM and the cells were immediately stimulated with 300 nM fMLP for 10 min at 37° C. LTB_4 biosynthesis was measured by reversed-phase HPLC (38); amounts of LTB_4 indicated represent the sum of LTB_4, 20-OH-LTB_4, and 20-COOH-LTB_4. The data shown are the mean ± SD of triplicate incubations from one of three representative experiments.

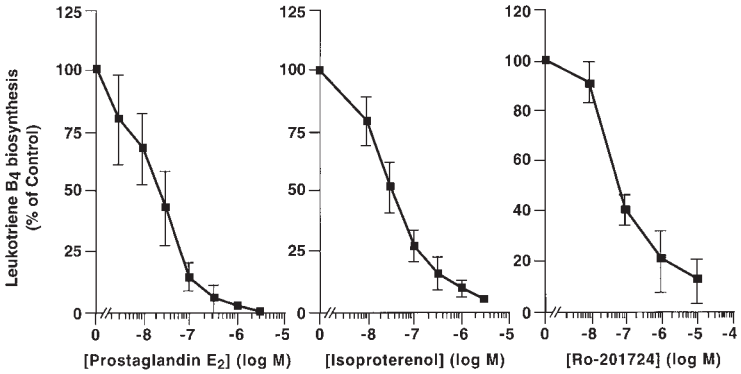

Figure 5. Effect of prostaglandin E_2 (PGE_2), isoproterenol, and RO-201724 on LTB_4 biosynthesis in activated neutrophils. Neutrophils (5×10^6/ml) were pretreated with GM-CSF and TNF-α for 30 min at 37° C, and then exposed to increasing concentrations of either PGE_2, isoproterenol, or RO-201724 in the presence of adenosine deaminase (0.1 U/ml) for 5 min before stimulation with 100 nM fMLP for a further 10-min incubation period at 37° C. LTB_4 biosynthesis was measured by reversed-phase HPLC; the data shown are the mean ± SEM of three separate experiments, each performed in triplicate.

vated neutrophils (14, 15). In the course of our investigations on the mechanisms of the inhibitory effect of CGS21680 on LTB_4 biosynthesis in neutrophils, we have obtained indirect but compelling evidence that elevation of intracellular cyclic AMP levels does result in a profound inhibition of AA release in activated human neutrophils. Indeed, using a variety of experimental conditions causing (through four distinct mechanisms) an elevation of intracellular cyclic AMP levels, it was observed that both LTB_4 biosynthesis and AA release were strongly and consistently inhibited (Figure 5 and data not shown). These different approaches include the use of neutrophil agonists acting through G protein-linked seven-transmembrane domain membrane receptors, namely PGE_2, isoproterenol, and CGS-21680; of agents that activate adenylate cyclase activity acting downstream of the membrane receptors, such as forskolin and cholera toxin; of agents that inhibit the type IV phosphodiesterase (present in neutrophils), such as RO-201724; or of membrane-permeable and phosphodiesterase-resistant analogs of cyclic AMP. Figure 5 illustrates inhibition of LTB_4 biosynthesis by agents that elevate intracellular cyclic AMP levels through three distinct mechanisms. Although these data do not provide information on how cyclic AMP downregulates AA release, they conclusively demonstrate that enhanced intracellular cyclic AMP levels result in the downregulation of AA release in activated neutrophils and, consequently, that cyclic AMP is an intracellular mediator of the inhibitory effect of A_{2a} receptor agonists on LTB_4 biosynthesis in human neutrophils.

BIOLOGICAL SIGNIFICANCE OF ADENOSINE-MEDIATED DOWNREGULATION OF LTB_4 BIOSYNTHESIS

It is well established that adenosine accumulates in leukocyte suspensions as a consequence of the extracellular breakdown of ATP (48); its accumulation in the extracellular milieu reaches concentrations that exert suppressive effects on neutrophil functions, including LTB_4 biosynthesis (49). Figure 6A shows that within 15 min, adenosine concentration increases by eightfold in a neutrophil suspension *in vitro*; Figure 6A also shows that the addition of adenosine deaminase in the incubation medium efficiently reduces adenosine concentrations in neutrophil suspensions. In the course of our investigations, adenosine deaminase was routinely added to neutrophil suspensions to eliminate endogenous adenosine from neutrophil incubations. The experiment depicted in Figure 6B conclusively demonstrates that adenosine generated in a neutrophil suspension profoundly downregulates the formation of LTB_4 on activation of neutrophils with agonists; indeed, the addition of either adenosine deaminase or the A_{2a} receptor antagonist 8-(3-chlorostyryl)caffeine (CSC) strikingly enhances the stimulated production of LTB_4 by the neutrophil suspensions, dem-

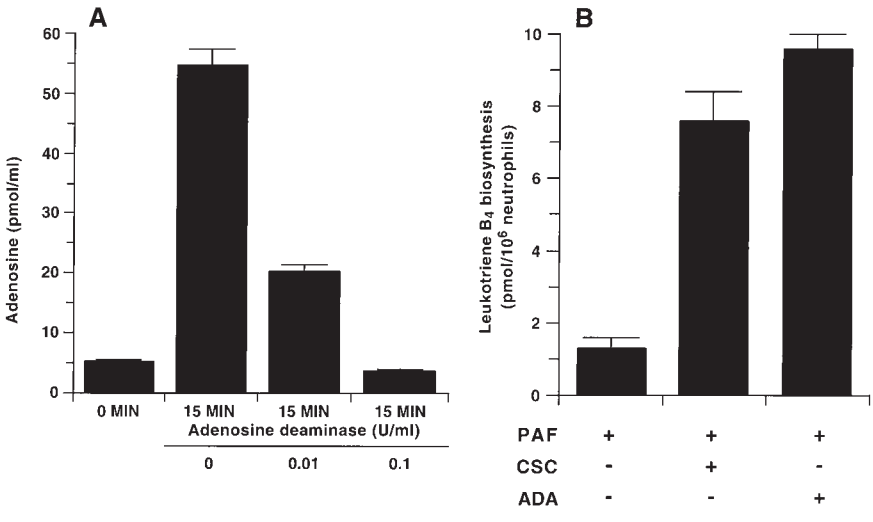

Figure 6. (*A*) Effect of adenosine deaminase (ADA) on adenosine accumulation in neutrophil suspensions. Freshly isolated neutrophils were resuspended in HBSS at a concentration of 20×10^6/ml; the adenosine content of the neutrophil suspension was measured either immediately (0 min) after the resuspension of neutrophils in HBSS, or 5 min after the addition (or not) of adenosine deaminase (0.01 and 0.1 U/ml) to neutrophil suspensions previously incubated for 15 min at 37° C. Adenosine was measured by liquid chromatography-mass spectrometry (electrospray ionization) as described previously (49). The data shown are the mean ± SD of triplicate incubations from one of three representative experiments. (*B*) Effect of the A_{2a} receptor antagonist chlorostyrylcaffeine (CSC) and adenosine deaminase (ADA) treatment of neutrophil suspensions on LTB_4 biosynthesis. Neutrophil suspensions in HBSS (10^7/ml) were preincubated with 700 pM GM-CSF plus 1.2 nM TNF-α for 30 min at 37° C and then treated with either 3 μM CSC or ADA (0.1 U/ml) for 5 min before stimulation with 0.6 μM PAF for 10 min at 37° C. LTB_4 biosynthesis was measured by reversed-phase HPLC. Amounts of LTB_4 indicated represent the sum of LTB_4, 20-OH-LTB_4, and 20-COOH-LTB_4; the data are the mean ± SD of triplicate incubations from one of three representative experiments.

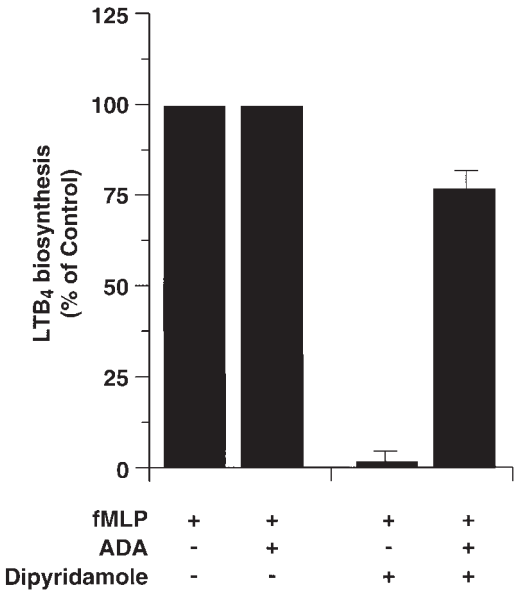

Figure 7. Reversal by adenosine deaminase (ADA) of the inhibitory effect of dipyridamole on LTB_4 biosynthesis in activated heparinized whole blood. Blood aliquots (1 ml) were pretreated with LPS (1 μg/ml) plus 1.2 nM TNF-α for 30 min at 37° C, in the presence or absence of 30 μM dipyridamole, then stimulated with 1 μM fMLP for 15 min. Control level (100%) of LTB_4 was 16.5 ± 1.0 ng/ml of plasma. LTB_4 was measured in plasma by reversed-phase HPLC as described previously (39). Data shown are the mean ± SD of triplicate determinations from one of three representative experiments.

onstrating that endogenous adenosine does suppress the formation of LTB_4 under these experimental conditions. In separate studies, it was shown that endogenous adenosine also suppressed LTB_4 biosynthesis in neutrophil suspensions in plasma (data not shown, *see* Reference 49). These data clearly demonstrate that endogenous adenosine is an efficient modulator of LTB_4 biosynthesis by neutrophils, and also suggest that in inflammatory exudates, rich in neutrophils, accumulation of adenosine may play a role in modulating the local generation of some important lipid mediators of inflammation. In whole blood, the concentration of extracellular adenosine is maintained at a low level by the efficient uptake of the autacoid by erythrocytes (50); this particular condition observed in whole blood facilitates the biosynthesis of LTB_4 on stimulation of blood with neutrophil agonists (37, 49). When dipyridamole, a substance known to inhibit the adenosine transporter on erythrocytes, was added to whole blood, the agonist-induced formation of LTB_4 was profoundly inhibited, an effect that was reversible by the addition of adenosine deaminase to blood (Reference 36 and Figure 7).

CONCLUDING REMARKS

In summary, our studies demonstrate the regulatory role of adenosine in ligand-stimulated LT biosynthesis by neutrophils and strongly emphasize that an elevated level of endogenous adenosine in physiological settings can have profound consequences on the ability of neutrophils to produce LTA_4, the direct precursor of the lipid mediators LTB_4, LTC_4, and lipoxins which have been shown to modulate phagocyte functional responses and inflammatory events. Thus, our observations strengthen the recently proposed hypothesis that adenosine is a natural antiinflammatory agent (16). Taken together, these observations and the previously reported inhibitory effects of adenosine on neutrophil and monocyte functions suggest that A_{2a} receptor agonists or agents that can regulate adenosine biosynthesis, metabolism, or transport may represent a novel class of potent antiinflammatory agents. In support of the hypothesis that the pharmacological regulation of adenosine may have therapeutic (antiinflammatory) applications, it was proposed that sulfasalazine and methotrexate, two drugs currently used in the treatment of inflammatory diseases (in particular methotrexate in rheumatoid arthritis), increase adenosine levels at inflammatory sites (16, 51); most importantly, these studies showed that leukocyte accumulation at inflammatory sites was diminished and that these effects of the drugs could be antagonized by adenosine deaminase or adenosine receptor antagonists (28). In view of the ability of adenosine to suppress LTB_4 biosynthesis by neutrophils (usually abundant in inflammatory exudates), it is tempting to speculate that the mechanism(s) by which these antiinflammatory agents act might include the inhibition of LTB_4-dependent extravasation of leukocytes. Further studies are needed to characterize the consequences of increasing adenosine levels on LTB_4-mediated inflammatory processes.

Finally, these studies clearly establish the occurrence of natural and biologically significant mechanisms of downregulation of LTB_4 biosynthesis in neutrophils; these observations also emphasize the importance of a comprehensive characterization of both stimulatory and inhibitory mechanisms that regulate the formation of the lipid mediators of inflammation. The understanding of these complex regulatory mechanisms will provide clues to the development of novel antiinflammatory approaches.

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