Inhibition of NADPH Oxidase Activation by 4-(2-Aminoethyl)-benzenesulfonyl Fluoride and Related Compounds*

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The elicitation of an oxidative burst in phagocytes rests on the assembly of a multicomponental complex (NADPH oxidase) consisting of a membrane-associated flavocytochrome (cytochrome b<sub>559</sub>), representing the reductant element responsible for the NADPH-dependent reduction of oxygen to superoxide (O<sub>2</sub> -), two cytosolic components (p47<sub>phox</sub>, p67<sub>phox</sub>), and the small GTPase Rac (1 or 2). We found that 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), an irreversible serine protease inhibitor, prevented the elicitation of O<sub>2</sub>- production in intact macrophages and the amphiphile-dependent activation of NADPH oxidase in a cell-free system, consisting of solubilized membrane or purified cytochrome b<sub>559</sub> combined with total cytosol or a mixture of recombinant p47<sub>phox</sub>, p67<sub>phox</sub>, and Rac1. AEBSF acted at the activation step and did not interfere with the ensuing electron flow. It did not scavenge oxygen radicals and did not affect assay reagents. Five other serine protease inhibitors (three irreversible and two reversible) were found to lack an inhibitory effect on cell-free activation of NADPH oxidase. A structure-function study of AEBSF analogues demonstrated that the presence of a sulfonyle fluoride group was essential for inhibitory activity and that compounds containing an aminoalkylbenzene moiety were more active than amidinobenzene derivatives. Exposure of the membrane fraction or of purified cytochrome b<sub>559</sub>, but not of cytosol or recombinant cytosolic components, to AEBSF, in the presence of a critical concentration of the activating amphiphile lithium dodecyl sulfate, resulted in a marked impairment of their ability to support cell-free NADPH oxidase activation upon complementation with untreated cytosol or cytosolic components. Kinetic analysis of the effect of varying the concentration of each of the three cytosolic components on the inhibitory potency of AEBSF indicated that this was inversely related to the concentrations of p47<sub>phox</sub> and, to a lesser degree, p67<sub>phox</sub>. AEBSF also prevented the amphiphile-elicited translocation of p47<sub>phox</sub> and p67<sub>phox</sub> to the membrane. These results are interpreted as indicating that AEBSF interferes with the binding of p47<sub>phox</sub> and/or p67<sub>phox</sub> to cytochrome b<sub>559</sub> probably by a direct effect on cytochrome b<sub>559</sub>.

The production of reactive oxygen radicals represents the major microbicidal mechanism of phagocytes (1). Oxygen radicals are also generated, in lesser amounts, by some nonphagocytic cells, sharing the enzymatic machinery characteristic of phagocytes (2) and, under certain conditions, by plant cells (3). Interest in reactive oxygen species has also been stimulated by accumulating evidence for their involvement in the pathogenesis of diseases, ranging from respiratory distress syndrome to ischemia-reperfusion injury in several organs (4).

The primordial oxygen radical produced by phagocytes is superoxide (O<sub>2</sub> -). It is generated, in response to appropriate stimuli, by NADPH-derived one-electron reduction of molecular oxygen, a reaction catalyzed by a membrane-bound heterodimeric flavocytochrome (cytochrome b<sub>559</sub>) (reviewed in Refs. 5–7). Cytochrome b<sub>559</sub> contains two redox centers, FAD and heme, and electron flow from NADPH to oxygen in initiated by the interaction of cytochrome with two cytosolic proteins, p47<sub>phox</sub> and p67<sub>phox</sub>, and the small GTPase, Rac1 or Rac2 (reviewed in Ref. 8). In the intact cell, this interaction is made possible by the stimulus-dependent translocation of the cytosolic components to the plasma membrane, leading to the assembly of what is known as the NADPH oxidase complex. Activation of NADPH oxidase, resulting in O<sub>2</sub>- generation, can be reproduced in vitro by a cell-free system consisting of either phagocyte membranes and cytosol (9, 10) or of a mixture of purified or recombinant components, exposed to a critical amount of an anionic amphiphile (arachidonate or SDS) (11, 12).

A number of inhibitors of the O<sub>2</sub>- generating NADPH oxidase have been described (reviewed in Ref. 13). The search for such inhibitors is propelled by two incentives: (a) to serve as tools for understanding the structure and mechanism of activation of the NADPH oxidase, and (b) their potential use as therapeutic agents in diseases associated with production of oxygen radicals at an inappropriate site or time. In most studies, inhibitors were tested for an effect on intact phagocytes. The main drawback of such an approach is that it does not permit a distinction between an effect on membrane receptors and signal transduction and a direct effect on the components of the NADPH oxidase complex. Alternative strategies rest on examining the effect of inhibitors on subcellular fractions originating from stimulated cells or on NADPH oxidase activation in the cell-

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free system. These latter approaches should allow the selection of inhibitors with a direct effect on the assembled complex or on individual components of the NADPH oxidase complex. So far, the only compound having gained wide acceptance as a relatively specific direct inhibitor of NADPH oxidase is diphenylene iodonium (14).

In the present paper we report that 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF; also known as Pefabloc SC), originally developed as an irreversible serine protease inhibitor, prevents the activation of the \( \text{O}_2^- \) generating NADPH oxidase in both intact stimulated macrophages and in cell-free systems. The effect is shared by some structurally related compounds, indicating that we are dealing with a new class of NADPH oxidase inhibitors. Evidence is presented in favor of the proposal that AEBSF interferes with the interaction of \( \text{p47}^{\text{phox}} \) and/or \( \text{p67}^{\text{phox}} \) with cytochrome \( b_{559} \), probably by chemical modification of cytochrome \( b_{559} \).

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**

AEBSF hydrochloride (≥99% pure), lithium dodecyl sulfate (LiDS), and common laboratory chemicals (at the highest purity available) were from Merck, 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSNH₂), 99% pure, was purchased from Aldrich. 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSAc), 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (MAEBSF), and 4-(aminido)-benzenesulfonyl fluoride hydrochloride (pABSF) were synthesized by Pentapharm Ltd., Basle, Switzerland. \( \alpha \)-Octyl-\( \beta \)-glucopyranoside (octyl glucoside) was a product of Pfannstiel Laboratories. Apritinin was obtained from Fluka. Guanosine 5′-3′-(thio)triphosphate (GTP-\( \gamma \)-S) was purchased from Boehringer. The following chemicals were obtained from Sigma: NADPH (tetrasodium salt, 95% pure), ferricytochrome \( c \) (from horse heart, 95% pure), superoxide dismutase (from bovine blood), xanthine, xanthine oxidase (from buttermilk), phosphor 12-myristate 13-acetate (PMA), \( \alpha \)-formyl-\( \alpha \)-Met-Leu-\( \alpha \)-Leu-Phe (FMLP), calcium ionophore A23187, phenylmethylsulfonyl fluoride (PMSF), 4-(aminido)-phenylmethylsulfonyl fluoride hydrochloride (pAPMSF), benzamidine hydrochloride, leupeptin hemisulfate salt, \( n \)-tosyl-l-phenylalanine chloromethyl ketone (TPCK), and 3,4-dichloroisoucoumarin (DCIC). Protease inhibitors were dissolved in water at a concentration at least 50-fold higher than the highest concentration used in NADPH oxidase inhibition assays, with the exception of PMSF, TPCK, and DCIC, which were dissolved in ethanol, methanol, and dimethyl sulfoxide, respectively.

**Preparation of Macrophages and Subcellular Fractions**

Macrophages were obtained from the peritoneal cavity of guinea pigs injected with mineral oil 5–6 days before cell harvest. The cells were washed and made erythrocyte-free, as described previously (10), and suspended in Earle’s balanced salt solution (containing 1.8 mM CaCl₂) supplemented with 90 \( \mu \)M ferricytochrome \( c \), in disposable spectrophotometer cuvettes. Inducers of an oxidative burst were added in a volume of 10 \( \mu \)l and the cuvettes placed in the thermostatted (37 °C) cuvette holder of a Uvikon 860 spectrophotometer (Kontron Instruments). Stimulus-dependent \( \text{O}_2^- \) production was assayed by the continuous recording of superoxide dismutase-inhibitable ferricytochrome \( c \) reduction, measured at 550 nm, and the maximal rate was calculated from the linear sector of the change in absorbance curve (23).

**Assays for Cell-free Activation of NADPH Oxidase**

**O\(_2\)** Production—Anionic amphiphile (LiDS)-activated \( \text{O}_2^- \) production in vitro was assayed by ferricytochrome \( c \) reduction in mixtures consisting of solubilized membrane and either total cytosol or a combination of \( \text{p47}^{\text{phox}} \), \( \text{p67}^{\text{phox}} \), and Rac1-GTP-\( \gamma \)-S, as described (10, 16). In some experiments, the membrane was replaced by purified relipidated cytochrome \( b_{559} \). Cell-free assays were performed either by the “one-step” method, in which activation by LiDS and NADPH-dependent \( \text{O}_2^- \) production occurred simultaneously, or by the “two-step” method (24) permitting at least partial, separation of the activation and catalytic stages of the reaction.

**NADPH Oxidation—LiDS-dependent NADPH oxidation by cell-free mixtures, of a composition identical to those utilized in the \( \text{O}_2^- \) production assays, was measured as described in Ref. 25.**

**Oxygen Consumption—LiDS- and NADPH-dependent oxygen consumption by cell-free mixtures, consisting of membrane and recombinant cytosolic components, was assayed with the aid of a Clark oxygen electrode (Yellow Springs Instruments), as described (26).**

**Translocation of \( \text{p47}^{\text{phox}} \) and \( \text{p67}^{\text{phox}} \) to the Membrane**

The translocation of \( \text{p47}^{\text{phox}} \) and \( \text{p67}^{\text{phox}} \) to the membrane fraction of macrophages was studied in the LiDS-activated cell free system. Macrophage membranes (equivalent to 1.6 × 10⁶ cells) were mixed with total macrophage cytosol (equivalent to 1.5 × 10⁷ cells) in a total volume of 1 ml of NADPH oxidase assay buffer (10), containing ferricytochrome \( c \), and incubated for 5 min at 24 °C, in the absence or presence of 185 \( \mu \)M LiDS (a concentration found to induce maximal \( \text{O}_2^- \) production under these conditions). The mixture was centrifuged at 15,800 × \( g \) for 30 min at 4 °C, and, after careful removal of the supernatant, the membrane pellet was resuspended in 1 ml of NADPH oxidase assay buffer and centrifuged at 15,800 × \( g \) for 15 min.

After removal of the supernatant, the resuspended membranes were resuspended in 30 \( \mu \)l of electrophoresis sample buffer containing 2% SDS, heated at 95 °C for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting, as described (19). The blots were probed with a mixture of goat polyclonal anti-recombinant \( \text{p47}^{\text{phox}} \) and anti-recombinant \( \text{p67}^{\text{phox}} \) antibodies (20, kind gifts of Dr. Thomas L. Leto, National Institutes of Health, Bethesda, MD).
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RESULTS

AEBSF Inhibits \( O_2^- \) Production by Intact Macrophages—\( O_2^- \) production can be elicited in guinea pig peritoneal macrophages by a variety of stimuli (27). We chose three stimuli (PMA, fMLP, and the calcium ionophore, A23187), known to activate NADPH oxidase in intact cells by distinct transductional mechanisms, and used these at concentrations found to elicit maximal \( O_2^- \) generation in cells in suspension at 37 °C. In the presence of AEBSF, a dose-dependent inhibition of \( O_2^- \) production elicted by 2 \( \mu M \) PMA was observed (Fig. 1A). When AEBSF was added just before the addition of PMA, the IC_{50} was close to 1 \( mM \) and inhibition was almost complete at 5 \( mM \) AEBSF. At the latter concentration, AEBSF also caused a total suppression of \( O_2^- \) production elicited by 1 \( mM \) fMLP or 10 \( \mu M \) A23187 (results not shown). This result was compatible with three interpretations: (a) AEBSF acts as a scavenger of \( O_2^- \); (b) it acts on a signal transduction path shared by all three stimuli; and (c) it has a direct effect on the NADPH oxidase complex.

AEBSF Does Not Scavenge \( O_2^- \)—We examined the effect of AEBSF on the ability to measure \( O_2^- \) generation by the xanthine-xanthine oxidase system, using the ferricytochrome \( c \) reduction assay. AEBSF (0.5–5 \( mM \)) did not interfere with the detection of \( O_2^- \) generated by xanthine (0.2 \( mM \)) and xanthine oxidase (0.018 units/ml); the difference between \( O_2^- \) detection in the absence or presence of AEBSF (up to 5 \( mM \)) was less than 10% of the control value.

AEBSF Inhibits \( O_2^- \) Production by a Cell-free System—NADPH oxidase can be activated in a cell-free system by certain anionic amphiphiles, a mechanism which circumvents the transductional pathways active in the intact phagocyte (9, 10). We, therefore, examined the effect of AEBSF on \( O_2^- \) production in several forms of cell-free activation systems. The simplest one consisted of solubilized macrophage membranes and unfractionated cytosol, both derived from unstimulated cells. AEBSF was found to exhibit a concentration-related, inhibitory influence on LiDS-induced \( O_2^- \) production, with an IC_{50} of \( 0.206 \pm 0.014 \) \( mM \) (\( n = 8 \)) (Fig. 1B). We next investigated the effect of AEBSF on a semi-recombinant cell-free system (11, 12), consisting of either solubilized macrophage membrane or purified cytochrome \( b_559 \), incorporated in phospholipid liposomes, combined with recombinant p47^phox^, p67^phox^, and Rac1-GTP^yS^S. AEBSF was found to act as an inhibitor of \( O_2^- \) production in this system, too, composed of four purified NADPH oxidase components in the virtual absence of any other protein (Fig. 1C). In the course of the latter experiments we became aware of an unexpected variability in the inhibitory potency of AEBSF which was not found in the cell-free system consisting of solubilized membrane and unfractionated cytosol. Analysis of this phenomenon revealed that, in the presence of a constant amount of membrane or purified cytochrome \( b_559 \), the inhibitory effect of AEBSF was inversely related to the concentration of \( p47^{phox} \). A more detailed exploration of the mechanism of this correlation is provided in the penultimate section.

To further ascertain that inhibition by AEBSF of \( O_2^- \) production in the cell-free system is due to a direct effect on NADPH oxidase, we examined the influence of AEBSF on two additional indicators of NADPH oxidase activation. These were LiDS-elicited NADPH oxidation (25) and LiDS- and NADPH-dependent oxygen consumption (26), by mixtures of membrane and cytosol or membrane and recombinant cytosolic components. We found that AEBSF suppressed NADPH oxidation in a concentration-related manner; when cell-free mixtures were analyzed in parallel by ferricytochrome \( c \) reduction, the IC_{50} of AEBSF values were identical in the two assays. Also, at 5 \( mM \), AEBSF inhibited oxygen consumption by 95%. We concluded that AEBSF acts by interacting with one or more of the components of the NADPH oxidase complex.

Inhibition by AEBSF Cannot Be Overcome by Excesses of Substrate, Cofactor, or Activator—The \( O_2^- \) generating NADPH oxidase has a \( K_m \) for NADPH of 20–40 \( \mu M \) (6) and the enzyme activated by amphiphile under cell-free conditions exhibits the
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FIG. 2. AEBSF inhibits NADPH oxidase activation and has little effect on electron transport. Solubilized macrophage membrane (4.5 pmol of cytochrome b_{559}) was incubated with total cytosol (equivalent to 4.5 × 10⁶ cells) in 100 µl of assay buffer containing 300 µM LiDS, for 5 min, in the absence or presence of AEBSF. Following this, the reaction volume was brought to 1 ml with assay buffer and O₂⁡/s/mol of cytochrome b_{559}. Results represent means ± S.E. of three experiments.

FIG. 3. Inhibition of NADPH oxidase activation by AEBSF is only partially reversible. Solubilized macrophage membrane (5.75 pmol of cytochrome b_{559}) was mixed with total cytosol (equivalent to 4.5 × 10⁶ cells) in 100 µl of assay buffer, containing 300 µM LiDS, and allowed to stand for 5 min, in the absence (1) or presence (2) of 2 mM AEBSF. This was followed by the addition of 0.9 ml of assay buffer, not containing LiDS, keeping the mixture for 3 min and initiating O₂⁡ production by the addition of 0.2 mM NADPH. In a parallel series of experiments, membrane/cytosol/LiDS mixtures were prepared as above, in the absence (3) or presence (4) of 2 mM AEBSF, but this was followed by the addition of 0.9 ml of assay buffer containing 120 µM LiDS and, after 3 min, by initiation of O₂⁡ production by the addition of 0.2 mM NADPH. To mixtures prepared in the absence of AEBSF (1 and 3), AEBSF was added at a concentration of 0.2 mM, together with the 0.9 ml of buffer, to reach a concentration of AEBSF equivalent to that present in samples 2 and 4, upon addition of 0.9 ml of buffer. The figure represents means ± S.E. of three experiments.

The same kinetic characteristics (9, 10). The effect of AEBSF (2 mM) on NADPH oxidase activation in the cell-free system was examined at concentrations of NADPH varying from 10 to 400 µM and analyzed by Michaelis-Menten plotting. This demonstrated that inhibition of AEBSF was noncompetitive with NADPH. Membrane solubilization and purification of cytochrome b_{559} results in a partial loss of FAD from the cytochrome (11, 19, 28), which explains the dependence of NADPH oxidase activation, in the cell-free system, on exogenous FAD (9). We, therefore, investigated whether AEBSF interfered with the reflavination of cytochrome b_{559}, by assessing the effect of varying the concentration of exogenous FAD on the inhibitory effect of AEBSF. We found that the degree of inhibition by AEBSF (IC₅₀) was independent of the concentration of exogenous FAD (from 10 to 100 µM). Finally, we explored the possibility that AEBSF competes with the activating amphiphile for interaction with a component of the NADPH oxidase complex. We found that increasing the concentration of LiDS, from the optimal activating range of 120–140 µM to up to 180 µM, did not reverse or reduce the inhibitory effect of AEBSF (see also Fig. 4).

AEBSF Interferes with Activation of NADPH Oxidase but Not with Electron Flow—Activation of NADPH oxidase, in intact cells and in broken cell preparations, is considered to be the result of the assembly of the individual components of the enzyme into a membrane-localized complex (reviewed in Ref. 8), this being followed by the induction of electron flow within the flavocytochrome b_{559} dimer. The “activation” and “electron flow” stages can be separated in a two-step cell-free activation assay (24). This consists of exposing a mixture of NADPH oxidase components to an optimal concentration of activating amphiphile in a small volume (100 µl) for a fixed time interval, in the absence of substrate NADPH, followed by 10-fold dilution and the initiation of electron flow by addition of NADPH. The 10-fold dilution results in a reduction in the concentration of amphiphile to a subactivating level and causes the virtual interruption of further assembly of the enzyme complex. We utilized this approach to identify the event affected by the inhibitor, by adding AEBSF either to the activation mixture at time 0 or after the completion of activation. As apparent in Fig. 2, AEBSF was a much more effective inhibitor when present during the assembly of the NADPH oxidase complex than when added at the completion of assembly. The finding that AEBSF was not totally inactive when added at the end of step one is probably due to the fact that some enzyme assembly continues taking place in the course of the second step. The ability of AEBSF to suppress O₂⁡ production when added to the components of the NADPH oxidase complex, in the absence of electron flow, also indicates that its mechanism of action is distinct from that of diphenylene iodonium, which acted as an inhibitor only under reducing conditions, such as those generated by

FIG. 4. The ability of AEBSF to prevent NADPH oxidase activation is not shared by other serine protease inhibitors. Solubilized macrophage membrane (2.52 pmol of cytochrome b_{559}) was combined with total cytosol (equivalent to 4.5 × 10⁶ cells) in 1 ml of assay buffer and activated with various concentrations of LiDS (40–180 µM) for 90 s, in the absence or presence of the indicated concentrations of serine protease inhibitors. O₂⁡ production was initiated by the addition of 0.2 mM NADPH. The figure was generated by the superposition of data from two characteristic experiments: one, comparing the effects of AEBSF, PMSF, and TPCK and the second, assessing the effect of DCIC. Each measurement was performed in triplicate and the values plotted on the y scale represent means ± S.E. of the amounts of O₂⁡ produced per assay mixture per min.
NADPH oxidase turnover (29).

Reversibility of Inhibition by AEBSF—Use was made of the two-step cell-free assay for determining whether inhibition of NADPH oxidase activation by AEBSF was reversible. For this purpose, activation mixtures were prepared in the absence and presence of a single concentration of AEBSF (2 mM) and incubated with an optimal concentration of LiDS for 5 min. Following this, the reaction mixtures were diluted in assay buffer to reduce the amphiphile concentration, and re-exposed or not to an activating concentration of LiDS for 3 min. The results illustrated in Fig. 3 demonstrate that re-exposure to LiDS of mixtures treated with 2 mM AEBSF partially reverses the inhibition. Thus, O$_2^.$ production by activation mixtures treated with AEBSF was 22.67 ± 1.12% of that of control preparations, whereas re-exposure of the AEBSF-treated preparations to LiDS led to resurgence of O$_2^.$ production, reaching 55.55 ± 1.99% of that measured in untreated preparations.

Specificity of AEBSF-mediated Inhibition—AEBSF was developed originally as an irreversible serine protease inhibitor (30, 31). It was, therefore, essential to establish whether its inhibitory effect on NADPH oxidase activation was related to its anti-protease activity. Hence, we examined the ability of five additional serine protease inhibitors to affect NADPH oxidase activation in the cell-free system. These were the irreversible inhibitors PMSF (0.1–2 mM) and TPCK (10–200 μM), the “mechanism based” irreversible inhibitor, DCIC (0.25 mM), and the reversible inhibitors aprotinin (0.3 μM) and leupeptin (20 μM). None of these exhibited an inhibitory effect on NADPH oxidase activation. We next explored the possibility that inhibition might be related to the concentration of activating amphiphile. As apparent in Fig. 4, PMSF, TPCK, and DCIC did not possess an inhibitory effect at any LiDS concentration whereas AEBSF was a potent inhibitor over the whole range of amphiphile concentrations. On the contrary, PMSF, TPCK, and DCIC had an enhancing effect on NADPH oxidase activation, at concentrations of LiDS below those required for maximal activation.

Structural Requirements for Inhibitory Action—We investigated the capacity of several analogues of AEBSF to interfere with the activation of NADPH oxidase. One group consisted of aminoethylbenzene derivatives and included: MAEBSF, identical to AEBSF except for the presence of an amino-linked methyl group; AEBSAc, a product of alkaline hydrolysis of AEBSF, found to be inactive as a protease inhibitor (31); and AEBSNH$_2$, in which an amide group replaces the fluoride found in AEBSF, also reported to lack protease inhibitory activity (32). As apparent in Fig. 5A, AEBSAc and AEBSNH$_2$ were totally inactive, whereas MAEBSF exhibited an inhibitory potency identical to that of AEBSF (IC$_{50}$ = 0.285 ± 0.005 mM; n = 3). The second group consisted of amidinobenzene derivatives. Among these, pAPMSF was reported to be a potent irreversible serine protease inhibitor (33), and benzamidine acts as a peptidase inhibitor (34). As seen in Fig. 5B, only pABSF was a moderate inhibitor of NADPH oxidase activation; its IC$_{50}$ (1.49 ± 0.05; n = 3) was 7.5-fold higher than that of AEBSF. We also examined the influence of an excess of the two inactive analogues, AEBSAc and AEBSNH$_2$, sharing the aminoethylbenzene structure with AEBSF, on inhibition of NADPH oxidase activation by AEBSF. Neither of the two analogues, at 5 mM, was capable of reducing the inhibitory effect of AEBSF, assayed at concentrations ranging from 0.1 to 5 mM. These results demonstrate that a sulfonyl fluoride group, adjacent to the benzene ring, is essential for inhibitory activity and that aminoalkylbenzene derivatives are more potent inhibitory
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Effect of treating individual components of the NADPH oxidase complex with AEBSF on their ability to support O$_2^-$ production in the cell-free system

Various components, at the concentrations shown in the footnotes, were incubated with 2 mM AEBSF for 5 min, at room temperature, in a volume of 100 µl, in the absence or presence of LiDS, at concentrations shown in the footnotes. This was followed by the addition of the missing untreated NADPH oxidase components at saturating concentration in 0.9 ml of assay buffer containing a concentration of LiDS, to result in a final concentration of 120 µM. Preparations, not exposed to AEBSF, were supplemented, at this stage, with 0.2 mM AEBSF, as a control for an eventual effect of AEBSF on electron flow. O$_2^-$ production was elicited 90 s later by the addition of 0.2 mM NADPH. Results are expressed as % inhibition of NADPH oxidase activation by relating the activity of mixtures containing a component treated with 2 mM AEBSF to that of control mixtures containing untreated component and 0.2 mM AEBSF. Results represent means ± S.E. of four experiments, for each situation. Negative values were obtained when O$_2^-$ production by a reaction mixture containing a component treated with AEBSF exceeded that of the control mixture containing the untreated component.

| Material or component treated with AEBSF | % Inhibition of NADPH oxidase activation in the reconstituted cell-free system |
|------------------------------------------|--------------------------------------------------------------------------------|
| Total solubilized membrane*              | Treatment in the absence of LiDS | Treatment in the presence of LiDS |
| Total cytosol a                         | 1.42 ± 1.50                      | 64.25 ± 4.78                      |
| Purified relipidated cytochrome b$_{559}$ c | -3.43 ± 1.16                     | 43.98 ± 2.27                     |
| p47$_{p67\text{phox}}$ d                  | -0.33 ± 2.14                     | 11.47 ± 10.27                    |
| Rac1-GTP$_{p8\text{S}}$ f                 | 2.76 ± 1.56                      | -0.48 ± 1.72                     |

* Solubilized membrane (2.4–4.5 pmol of cytochrome b$_{559}$). In 100 µl, was exposed to AEBSF in the absence or presence of 300 µM LiDS. It was complemented with cytosol (equivalent to 4.5 × 10$^6$ cells) in 0.9 ml.

a Cytosol (equivalent to 4.5 × 10$^6$ cells), in 100 µl, was exposed to AEBSF in the absence or presence of 120 µM LiDS. It was complemented with solubilized membrane (4.5 pmol of cytochrome b$_{559}$), in 0.9 ml.
b Purified cytochrome b$_{559}$ (4.92 pmol), in 100 µl, was exposed to AEBSF in the absence or presence of 150 µM LiDS. It was complemented with cytosol (equivalent to 6 × 10$^6$ cells), in 0.9 ml.
c P47$_{p67\text{phox}}$ (12.2 pmol), in 100 µl, was exposed to AEBSF in the absence or presence of 140 µM LiDS. It was complemented with the missing components, in 0.9 ml, at the following final concentrations: solubilized membrane (2.4 nm cytochrome b$_{559}$), p67$_{p67\text{phox}}$ (6 nm), and Rac1-GTP$_{p8\text{S}}$ (100 nm).
d p67$_{p67\text{phox}}$ (6 pmol), in 100 µl, was exposed to AEBSF in the absence or presence of 140 µM LiDS. It was complemented with the missing components in 0.9 ml, at the following final concentrations: solubilized membrane (2.4 nm cytochrome b$_{559}$), p47$_{p47\text{phox}}$ (12.2 nm), and Rac1-GTP$_{p8\text{S}}$ (100 nm).
e p47$_{p47\text{phox}}$ (6 pmol), in 100 µl, was exposed to AEBSF in the absence or presence of 300 µM LiDS. It was complemented with the missing components in 0.9 ml, at the following final concentrations: solubilized membrane (2.4 nm cytochrome b$_{559}$), p47$_{p47\text{phox}}$ (12.2 nm), and p67$_{p67\text{phox}}$ (6 nm).

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Identification of the Molecular Target of AEBSF—We next investigated whether interaction between AEBSF and a specific NADPH oxidase component can be demonstrated. The basic design of these experiments was to expose subcellular fractions or individual NADPH oxidase components to AEBSF for a fixed time interval in a small volume, followed by 10-fold dilution into assay buffer containing the untreated complementary fraction or a mixture of the other, untreated NADPH oxidase components, activating LiDS and NADPH. Dilution resulted in a reduction in AEBSF concentration to subinhibitory levels, limiting its effect to the component initially exposed to the compound. We examined the effect of AEBSF on total membrane, total cytosol, relipidated cytochrome b$_{559}$, and recombinant p47$_{p47\text{phox}}$, p67$_{p67\text{phox}}$, and Rac1-GTP$_{p8\text{S}}$. Each of these was exposed or not to AEBSF in the absence or presence of LiDS, the maximal concentration of amphiphile being chosen not to exceed that permitting the recovery of NADPH oxidase activity, upon complementation with untreated components.

As apparent in Table I, exposure of total solubilized membrane or purified and relipidated cytochrome b$_{559}$ to AEBSF, in the presence but not in the absence of LiDS, resulted in marked inhibition of their capacity to support O$_2^-$ production upon complementation with cytosol and activation by LiDS. Total cytosol as well as p47$_{p47\text{phox}}$ and Rac1 were insensitive to treatment by AEBSF; a minor effect of AEBSF on p67$_{p67\text{phox}}$ was evident but this result was difficult to interpret because of considerable interexperimental variability and because, among all NADPH oxidase components, p67$_{p67\text{phox}}$ was the most sensitive to denaturation by LiDS.

The ability of AEBSF to inactivate cytochrome b$_{559}$ was investigated in more detail by assessing the dose-dependence of the inactivation of solubilized membrane by AEBSF, in the presence and absence of LiDS. As seen in Fig. 6, AEBSF inhibited the capacity of the membrane to support O$_2^-$ production only in the presence of LiDS, with an IC$_{50}$ of 2.18 mm.

AEBSF Interferes with the Interaction Between Cytochrome b$_{559}$ and p47$_{p47\text{phox}}$ and/or p67$_{p67\text{phox}}$—This series of experiments was initiated as an extension of the preliminary finding that, in the presence of a constant amount of cytochrome b$_{559}$, the inhibitory effect of AEBSF could be overcome by increasing the concentration of cytosolic component p47$_{p47\text{phox}}$. The consensus opinion is that an essential event in the assembly of the NADPH oxidase complex is the binding of p47$_{p47\text{phox}}$ to cytochrome b$_{559}$ (reviewed in Ref. 8). In light of the results described in the preceding section, it seemed likely that AEBSF competes with p47$_{p47\text{phox}}$ for interaction with cytochrome b$_{559}$.

We, therefore, investigated the influence of varying the concentration of p47$_{p47\text{phox}}$ on the inhibition of NADPH oxidase activation by AEBSF, in the presence of a constant amount of membrane and saturating concentrations of p67$_{p67\text{phox}}$ and Rac1-GTP$_{p8\text{S}}$ (Fig. 7A). In additional experiments, we varied the concentrations p67$_{p67\text{phox}}$ (Fig. 7B) and Rac1-GTP$_{p8\text{S}}$ (Fig. 7C), in the presence of saturating concentrations of the other two cytosolic components. In all experiments, three concentrations of cytosolic component were chosen, two of which were on the ascending slope of previously established dose-response curves, whereas the third one represented a saturating concentration.

The data were analyzed by plotting the "concentration of AEBSF" (on a logarithmic scale) against "inhibition of NADPH oxidase activation" and IC$_{50}$ of AEBSF values, for each concentration of cytosolic component, were calculated. As seen in Fig. 7A, when p47$_{p47\text{phox}}$ was present in sub saturating concentrations, sigmoid curves were generated and IC$_{50}$ values for AEBSF decreased with decreasing concentrations of p47$_{p47\text{phox}}$. IC$_{50}$ values were moderately reduced by lowering the concentration of p67$_{p67\text{phox}}$ (Fig. 7B) and not significantly affected by varying the concentration of Rac1-GTP$_{p8\text{S}}$ (Fig. 7C). These results are compatible with the hypothesis that AEBSF interferes with the binding of p47$_{p47\text{phox}}$ to cytochrome b$_{559}$ but we
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FIG. 6. Treatment of the membrane fraction with AEBSF results in impairment of its ability to cooperate with recombinant cytosolic components in NADPH oxidase assembly. Solubilized membrane (3.45 pmol of cytochrome b$_{559}$) was exposed or not to various concentrations of AEBSF (0.5–10 mM), in the presence or absence of 300 µM LiDS, for 5 min at room temperature, in a total volume of 100 µl. This was followed by the addition of p47$_{phox}$ (24 nM), p67$_{phox}$ (19.8 nM), and Rac1-GTP$_{S}$ (108 nM) in 0.9 ml of assay buffer containing 120 µM LiDS, bringing the total volume of the assay to 1 ml, and the mixture was kept for a further 90 s at room temperature, at which time O$_{2}$ production was elicited by the addition of 0.2 mM NADPH. Reaction mixtures which contained solubilized membrane not pre-exposed to AEBSF were supplemented with AEBSF, at one-tenth of the concentration added to the membranes, at the stage of adding the cytosolic components, as a control for an effect of AEBSF on cytosolic components or electron flow. The figure illustrates the results of a typical experiment, with each measurement being performed in duplicate. For each measurement, the results are expressed as % inhibition of NADPH oxidase activation by relating NADPH oxidase activity of mixtures, containing membrane pretreated with AEBSF, to that of control preparations containing untreated membrane interacting with cytosolic components in the presence of AEBSF at 10-fold lower concentration. NADPH oxidase activity in control preparations was 99.3 mol of O$_{2}$/s/mol of cytochrome b$_{559}$, when the membrane was pretreated with LiDS, and 91.5 mol of O$_{2}$/s/mol of cytochrome b$_{559}$, when the membrane was not pretreated with LiDS.

cannot rule out the possibility that AEBSF also inhibits a direct interaction between p67$_{phox}$ and cytochrome b$_{559}$ (see “Discussion”).

The hypothesis that AEBSF acts as a competitive inhibitor of p47$_{phox}$ was further tested by performing a Michaelis-Menten analysis of the effect of AEBSF on the activation of NADPH oxidase at four concentrations of p47$_{phox}$ (14, 21, 28, and 35 nM), all within the ascending slope of the concentration-response curve. As seen in the Lineweaver-Burk plot in Fig. 8, AEBSF, in the concentration range of 0.5 to 2 mM, behaves as a competitive inhibitor with respect to p47$_{phox}$. At concentrations of AEBSF exceeding 2 mM, a noncompetitive or mixed type of inhibition pattern was obtained. Similar kinetic analysis of the effect of AEBSF on NADPH oxidase activation at varying concentrations of either p67$_{phox}$ or Rac1-GTP$_{S}$ revealed noncompetitive or mixed type inhibition patterns.

AEBSF Prevents Amphiphile-dependent Translocation of p47$_{phox}$ and p67$_{phox}$ to the Membrane—Activation of NADPH oxidase in intact cells is accompanied by translocation of fractions of p47$_{phox}$ and p67$_{phox}$ to the plasma membrane (35). The anchoring of both cytosolic components to the membrane is dependent on the presence of cytochrome b$_{559}$ (36). Also, translocation of p67$_{phox}$ to the membrane occurs only in the presence of p47$_{phox}$ (36), suggesting that at least one of the functions of p47$_{phox}$ is to serve as an escort protein for p67$_{phox}$. It was, therefore, of interest to investigate the effect of AEBSF on the translocation of cytosolic components to the membrane in the cell-free system (37). As seen in Fig. 9, the addition of AEBSF to mixtures of macrophage membranes and cytosol, activated by a concentration of LiDS to result in maximal NADPH-dependent O$_{2}$ production, markedly inhibited the translocation of both p47$_{phox}$ and p67$_{phox}$ to the membrane. Parallel testing of the sedimented membranes, derived from cell-free mixtures activated in the absence and presence of 4.5 mM AEBSF, for O$_{2}$ production, showed that lack of translocation was accompanied by an 82% inhibition of NADPH oxidase activation. As apparent in Fig. 9B, translocation of p47$_{phox}$ and p67$_{phox}$ (and its inhibition by AEBSF) was strictly dependent on the presence of membranes and was not the result of nonspecific precipitation of cytosolic components by LiDS (35).
DISCUSSION

We found that AEBSF, originally developed as a watersoluble irreversible serine protease inhibitor of relatively low toxicity, interferes with the activation of the \( O_2^- \) generating NADPH oxidase both in intact macrophages and in cell-free systems in a concentration-dependent manner. A remarkable feature of this inhibition is that it affects all forms of cell-free activation, from broken cell systems, consisting of total membrane and unfractionated cytosol, to mixtures of purified cytochrome \( b_{559} \) and three recombinant cytosolic components. While this paper was in preparation, we became aware of the report by Remold-O’Donnell and Parent (38), describing the inhibitory effect of 2 mM AEBSF on \( O_2^- \) production by human neutrophils in response to PMA and opsonized zymosan. AEBSF (0.1–0.2 mM) was recently found to block priming of human monocytes for enhanced \( O_2^- \) production by intact phagocytes. Results of such experiments have indicated, indeed, that AEBSF affects NADPH oxidase activity in the cell-free system; 

There is a large body of literature linking the elicitation of an oxidative burst to the activation of cellular proteases (summarized in Ref. 39). Some of the evidence for such a connection is based on the effect of synthetic or natural protease inhibitors, particularly serine protease inhibitors, on oxygen radical production by intact phagocytes. Results of such experiments have to be interpreted with caution since it became evident that the effects of protease inhibitors on \( O_2^- \) production were frequently unrelated to their antiproteolytic activity (39–42). Our results indicate, indeed, that AEBSF affects NADPH oxidase activation, at least in cell-free preparations, by a mechanism unrelated to its protease inhibitory activity. This claim is supported by the following arguments: (a) five other serine protease inhibitors (PMSF, TPCK, DCIC, leupeptin, and aprotinin) were found to be inactive when tested under identical conditions (it is of interest that DCIC which, like AEBSF, was found to block \( O_2^- \) production by intact neutrophils (38), did not inhibit NADPH oxidase activation in the cell-free system); (b) among protease inhibitors, structurally related to AEBSF (PMSF and \( p \)APMSF), there was no relation between NADPH oxidase activation inhibitory and antiproteolytic activities, and (c) AEBSF was inhibitory in a cell-free system composed exclusively of purified native and recombinant proteins, and, therefore, free of contaminating proteases.

The inhibitory effect of AEBSF on NADPH oxidase activation exhibited a pronounced structural specificity. A number of derivatives of benzylamine and benzamidine, to which categories AEBSF is related, were shown to function as competitive serine protease inhibitors (43). The presence of a reactive sulfon fluoride group on the aromatic ring generates compounds capable of forming a covalent bond with their target enzymes (30, 44). We found that, among four compounds sharing an aminoethylbenzene moiety (AEBSF, AEBSAc, AEBSNH\(_2\), and MAEBSF), only AEBSF and MAEBSF, which possess a reactive sulfon fluoride group, were inhibitory. Among three amidinobenzene derivatives (benzamidine, \( p \)APMSF, and \( p \)APMSF) only \( p \)APMSF, possessing a sulfon fluoride group adjacent to the benzene ring exhibited moderate NADPH oxidase inhibitory activity. PMSF and TPCK, two serine protease inhibitors bearing limited structural similarity to AEBSF, but lacking the basic amino group present in AEBSF, were found to be inactive in the cell-free system. We conclude that two structural elements are the minimal prerequisites for the possession of NADPH oxidase inhibitory property: a positively charged aminalkyl moiety and a reactive sulfon fluoride group.

The search for the molecular target of AEBSF led to the conclusion that this is, most likely, cytochrome \( b_{559} \). This pro-
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posal is based principally on the finding that treatment of either total membrane fraction or purified and relipidated cytochrome b$_{559}$ with AEBSF, in the presence of a critical concentration of LiDS, resulted in a dose-dependent reduction in their ability to cooperate with untreated components in the assembly of NADPH oxidase. Neither total cytosol nor any of the three cytosolic components were sensitive to AEBSF to a comparable degree. A remarkable feature of this effect is its absolute dependence on the simultaneous presence of an anionic amphiphile. The amphiphile may make the liposome-enclosed cytochrome b$_{559}$ more accessible to AEBSF or may induce a conformational change in cytochrome b$_{559}$ itself, normally associated with the process of activation, but also leading to exposure of an AEBSF-binding domain. Evidence for a direct effect of anionic amphiphiles, at NADPH oxidase activating concentrations, on cytochrome b$_{559}$ was recently presented by the demonstration that arachidonate and SDS enhancing concentrations, on cytochrome b$_{559}$, results in a dose-dependent reduction in the translocation of both p47$^{phox}$ and p67$^{phox}$ and Rac1 plasmid in E. coli and S. Hanft for word processing.

Further exploration of the mechanism of inhibition of NADPH oxidase by AEBSF in particular and by aminooalkylbenzenesulfonyl fluorides in general is of both theoretical and practical relevance. Thus, it should lead to a better understanding of the molecular basis of the assembly of the NADPH oxidase complex and to the design of drugs to be used in circumstances associated with the undesired production of oxygen radicals. This latter aim, however, is dependent on the clarification of the relevance of effects found in the cell-free system to the situation in intact cells. Nevertheless, the fact that AEBSF is a stable, water-soluble and nonreactive compound, possessing both NADPH oxidase suppressory and anti-proteolytic properties, makes it an interesting model compound for the design of antiinflammatory drugs.

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