Hydroperoxide-induced Increases in Intracellular Calcium Due to Annexin VI Translocation and Inactivation of Plasma Membrane Ca\(^{2+}\)-ATPase*

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Oxidative stress can cause changes in intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) that resemble those occurring under normal cell signaling. In the alveolar macrophage, hydroperoxide-induced elevation of [Ca\(^{2+}\)]\(_i\) modulates the respiratory burst and other important physiologic functions. The source of Ca\(^{2+}\) released by hydroperoxide is intracellular but separate from the endoplasmic reticulum pool released by receptor-mediated stimuli (Hoyal, C. R., Gozal, E., Zhou, H., Foldenauer, K., and Forman, H. J. (1996) Arch. Biochem. Biophys. 326, 166–171). Previous studies in other cells have suggested that mitochondria are a potential source of oxidant-induced [Ca\(^{2+}\)]\(_i\) elevation. In this study we have identified another potential source of hydroperoxide-releasable intracellular calcium, that bound to annexin VI on the inner surface of the plasma membrane. Translocation of annexin VI from the membrane during exposure to \(t\)-butyl hydroperoxide matched elevation of [Ca\(^{2+}\)]\(_i\), as a function of time and \(t\)-butyl hydroperoxide concentration. The translocation was possibly due to a combination of ATP depletion and oxidative modification of membrane lipids and proteins. A sustained increase in [Ca\(^{2+}\)]\(_i\), occurring >50 pmol/10\(^6\) cells (50 \(\mu\)M under these conditions) appeared to be a consequence of membrane Ca\(^{2+}\)-ATPase dysfunction. These results suggest that exposure to oxidative stress results in early alterations to the plasma membrane and concomitant release of Ca\(^{2+}\) into the cytosol. In addition it suggests a mechanism for participation of annexin VI translocation that may underlie the alterations in macrophage function by oxidative stress.

Exposure of alveolar macrophages to oxidative stress has been shown to cause alterations in receptor-mediated signaling for the respiratory burst (1–3), modulation of phagocytosis and chemotaxis (4–7), and enhanced phospholipase \(A_2\) activity (8) with production of inflammatory mediators. Previously we have shown that release of intracellular calcium following exposure to hydroperoxides modulates the respiratory burst of alveolar macrophages (2, 9). The intracellular calcium pool is not released from the endoplasmic reticulum, and the endoplasmic reticulum remains replete and passively releasable by thapsigargin during and after the oxidant exposure. Calcium has a dual effect in modulating the signaling for the respiratory burst; enhancement with transient [Ca\(^{2+}\)]\(_i\), elevation and inhibition with sustained [Ca\(^{2+}\)]\(_i\) elevation. Transient elevation of [Ca\(^{2+}\)]\(_i\), (observed with <50 \(\mu\)M tBOOH) also correlates with reversible oxidation of NADPH, while sustained elevation of [Ca\(^{2+}\)]\(_i\) correlates with irreversible oxidation of NADH (10). We have previously shown that concentrations of hydroperoxide in excess of 50 \(\mu\)M also cause mitochondrial membrane depolarization (11). This suggests an alteration would occur in the ability of mitochondria to take up and retain Ca\(^{2+}\). Nevertheless, mitochondria do not store significant amounts of releasable Ca\(^{2+}\) (12) but rather modulate release from the IP\(_3\)-sensitive pool during signaling (13, 14). Thus, it is unlikely that the initial source of calcium released by hydroperoxide was from the mitochondria.

Another potential source of calcium is that bound to the plasma membrane. Annexin VI is a protein primarily found in alveolar macrophages in the lung, which binds to the plasma membrane in a calcium-dependent manner (15–18). In the present study we proposed that oxidative stress would alter the interaction of annexin VI with the membrane so that both the protein, and as a consequence Ca\(^{2+}\), would be released into the cytosol. We also examined whether the sustained elevation of [Ca\(^{2+}\)]\(_i\), could result from irreversible dissociation of annexin VI or inhibition of the plasma membrane Ca\(^{2+}\)-ATPase. Our results suggest that a combination of annexin VI translocation, Ca\(^{2+}\) pump inhibition and resultant changes in [Ca\(^{2+}\)]\(_i\) may underlie several functional alterations observed in alveolar macrophages during oxidative stress.

MATERIALS AND METHODS

Reagents—ADP, EGTA, DTT, phenylmethylsulfonyl fluoride, tBOOH, peptatin A, iodoacetamide, Hepes, Tris, and other buffer salts were purchased from Sigma. Tween 20 was purchased from Pierce. Indo-1 and fluo-3 were purchased from Molecular Probes, Inc. (Eugene, OR). Antibody to annexin VI (1:1000) was obtained from Zymed Laboratories, Inc. (San Francisco, CA) Biotin-conjugated goat anti-mouse antibody (1:5000) was purchased from Boehringer Mannheim. Immobilon-P transfer membranes were purchased from Millipore Corp. (Bedford, MA)

Preparation of Alveolar Macrophages—Alveolar macrophages are obtained by bronchoalveolar lavage from specific pathogen-free, 250–350-g Sprague-Dawley male rats (CRL:CD\(\times\)D::BR, Charles River, Inc., Wilmington, MA) using a previously described method (19). Briefly, the lungs are lavaged with 100 ml of sodium phosphate (0.01 M)-buffered saline. The lavage fluid was filtered through a 37-\(\mu\)m nylon cloth, and the filtrate is centrifuged for 10 min at 1000 \(\times\)g. Macrophages were resuspended and stored at 4°C in Krebs-Ringer-
phosphate buffer (KRPH), pH 7.4, containing 10 mM Hepes, 125 mM NaCl, 5 mM KCl, 1 mM MgSO\textsubscript{4}, 10 mM sodium phosphate, 5 mM glucose, 1.3 mM CaCl\textsubscript{2}). This preparation yields about 10\textsuperscript{7} cells/rat, of which >98% are viable alveolar macrophages.

**Spatial Localization of Ca\textsuperscript{2+} Transients—**Alveolar macrophages (5 × 10\textsuperscript{6}) were incubated at 37 °C in KRPH for 10 min. 5 × 10\textsuperscript{6} macrophages in 5 ml of buffer were exposed to buffer, or 25 μM to 1 mM tBOOH for 5 min. The macrophages were then centrifuged for 5 min at 1000 × g, and the pellets were resuspended in 1 ml of buffer containing 20 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml peptatin A, 10 μM iodoacetamide, 1 mM MgCl\textsubscript{2}, and 1 mM CaCl\textsubscript{2} (or 1 mM EGTA as a control). The cells were then disrupted with an Artek sonic dismembrator (Artek Systems Corp., Farmingdale, NY). The homogenates were centrifuged at 4000 × g for 15 min (4°C), and the post nuclear supernatants were centrifuged at 105,000 × g for 40 min (4°C) using a fixed-angel Beckman T50 rotor.

The 105,000 × g pellet was collected, and the supernatant was centrifuged at 105,000 × g for 90 min (4°C). The 105,000 × g pellet was collected, and the remaining cytosol was concentrated in a Speed Vac concentrator, Savant Instruments (Farmingdale, NY). The protein concentrations of each pellet were determined using the method by Lowry, and samples were frozen until analyzed.

**Gel Electrophoresis and Immunoblotting—**Proteins were resolved on 10% polyacrylamide Tris-glycine gels Novex (San Diego, CA) and electrophoresed onto Immobilon-P transfer membranes. The blots were soaked for 30 min in a blocking solution made of 5% nonfat dry milk in TBS. Membranes were incubated overnight with the anti-annexin VI (1:5000) for 2 h then washed in TBS-Tween. Membranes were then incubated overnight with the anti-annexin VI (1:5000) for 2 h then washed in TBS-Tween. Membranes were then washed in TBS. Membranes were incubated overnight with the anti-annexin VI (1:5000) for 2 h then washed in TBS-Tween. Membranes were then washed in TBS containing VECTASTAIN elite ABC, a secondary detection system Vector Laboratories, Inc. (Burlingame, CA) for 30 min, then washed in TBS-Tween. The proteins were visualized with the Amersham ECL detection system.

**Adenosine 5’-triphosphate Assay—**Alveolar macrophages (2 × 10\textsuperscript{6}) were isolated by centrifugation, and the mitochondrial uncoupler, 1700 (10 ng/ml prepared with 10 ng/ml oligomycin) for 5 and 15 min. The concentration of cellular ATP was determined using a bioluminescent ATP assay kit (Sigma). Briefly, following the incubation cells were centrifuged and resuspended in 500 μl of somatic cell ATP-releasing reagent and stored at 4 °C. To 100 μl of each sample, 100 μl of bioluminescent ATP assay mix were added, and the mixture was immediately measured for phosphorescence. The samples were then assayed for protein content, and the data are expressed as concentration of micromoles of ATP/g of protein.

**Effects of Dithiothreitol on [Ca\textsuperscript{2+}] and Ca\textsuperscript{2+} Efflux—**Alveolar macrophages (5 × 10\textsuperscript{5}) were incubated with 0.1% DTT for 30 min at 37 °C. Cells were stirred continuously and the cuvette holder thermostated at 37 °C. Fluorescence was measured with a Hitachi fluorescence spectrophotometer (excitation 355 nm and emission at 400 and 450 nm). Using the dissociation constant for Indo-1 and the ratio of the emission at 400 to 450 nm, R, [Ca\textsuperscript{2+}], was determined where:

\[
[Ca^2+]=K_d \cdot \frac{(R-R_{min}/R_{max}) \cdot R}{S_0/S_3}
\]

as defined by Grynkiewicz et al. (20) using the dissociation constant for Indo-1 (250 nm) and the value for Ca\textsuperscript{2+}-free indicator fluorescence divided by Ca\textsuperscript{2+}-bound indicator fluorescence, (S_3/S_0), as determined experimentally for our system. The Ca\textsuperscript{2+} ionophore, ionomycin (10 μM), was added to cells in the presence of 1.3 mM CaCl\textsubscript{2} to determine the maximum fluorescence (R_{max}) for measurements made with Indo-1.

**RESULTS**

**Spatial Localization of Initial Calcium Signal during Exposure to Hydroperoxide or ADP—**Confocal microscopy was used to identify the spatial localization of initial increases in [Ca\textsuperscript{2+}], following exposure to ADP, a receptor-mediated stimulant of the respiratory burst (21), or tBOOH. Exposure to 100 μM ADP initiates an increase in [Ca\textsuperscript{2+}], from internal stores of calcium, known to be the endoplasmic reticulum (3, 9). Fig. 1A depicts the line scan images over the first 76 s of the IP\textsubscript{3}-mediated signal. In contrast, exposure to the hydroperoxide tBOOH results in an increase in intracellular Ca\textsuperscript{2+} that began from the membrane as seen in Fig. 1B. This difference in location confirms that the calcium release was not from the endoplasmic reticulum. The cytoplasm immediately beneath the inner face of the plasma membrane is devoid of organelles suggesting that the calcium store released was membrane bound.
shown). Inclusion of EGTA and absence of Ca\(^{2+}\) indicated that the vast majority of annexin VI was bound to the membrane. The control was untreated macrophages which upon separation revealed that the majority of annexin VI was membrane-associated; the crude particulate with which the cytoskeleton is associated; and C (cytosol). The control was untreated macrophages which upon separation revealed that the majority of annexin VI was membrane-associated. Immunochemistry, using the same antibody, indicated that the vast majority of annexin VI was bound to the plasma membrane in control alveolar macrophages (not shown). Inclusion of EGTA and absence of Ca\(^{2+}\) in the lysis buffer did not result in a major movement of annexin VI into the cytosol but did result in an increase of annexin VI in the 20,000 \(\times\) g fraction. Following tBOOH treatment, annexin VI increased in the cytosol, but no change occurred in the 20,000 \(\times\) g fraction. Thus, the translocation of annexin VI into the cytosol with tBOOH was different from that caused by removal of Ca\(^{2+}\).

Translocation of Annexin VI—The subcellular distribution of annexin VI was investigated in order to determine if annexin VI translocates during oxidative stress. Fig. 2 shows the distribution of annexin VI in alveolar macrophages. The macrophages were lysed in the absence of detergents and separated into three fractions: the 20,000 \(\times\) g pellet, considered to be the crude particulate with which the cytoskeleton is associated; the 105,000 \(\times\) g pellet, considered to be membrane: and C, cytosol. The control was untreated macrophages which upon separation revealed that the majority of annexin VI was membrane-associated. Immunochemistry, using the same antibody, indicated that the vast majority of annexin VI was bound to the plasma membrane in control alveolar macrophages (not shown). Inclusion of EGTA and absence of Ca\(^{2+}\) in the lysis buffer did not result in a major movement of annexin VI into the cytosol but did result in an increase of annexin VI in the 20,000 \(\times\) g fraction. Following tBOOH treatment, annexin VI increased in the cytosol, but no change occurred in the 20,000 \(\times\) g fraction. Thus, the translocation of annexin VI into the cytosol with tBOOH was different from that caused by removal of Ca\(^{2+}\).

Fig. 3 depicts the concentration dependence of annexin VI translocation into the cytosol during exposure to hydroperoxides at concentrations resulting in transient or sustained increase in \([Ca^{2+}]\). There was a dramatic increase in annexin VI in the cytosol upon exposure to 25 \(\mu M\) tBOOH that maximizes at 100 \(\mu M\) tBOOH. Treatment with a lethal level of hydroperoxide (1 mM) does not increase annexin VI translocation into the cytosol over the 100 \(\mu M\) concentration. Further investigation into the translocation of annexin VI following exposure to hydroperoxide shows that translocation increases not only as a function of concentration, but also is dependent upon the time of hydroperoxide exposure. This suggests that the increase and decrease in intracellular calcium during exposure to hydroperoxides is occurring through at least two different mechanisms. This blot is representative of three separate experiments. Densitometry represents percentage cytosol control.

[Ca\(^{2+}\)], correlated with reversible oxidation of NADPH. Exposure to concentrations of hydroperoxide, in the range of 50 to 100 \(\mu M\), resulted in sustained oxidation of NADH (10). The NAD\(^+\) produced could be re-reduced after disruption of the cells. Thus, there was no degradation of the pyridine nucleotide pool under this sublethal oxidative stress. To determine if the mitochondria were playing a role in the uptake and release of calcium, the effects of mitochondrial uncouplers on tBOOH-mediated calcium release were explored. Pretreatment with the uncoupler, 1799, or inhibitor, KCN, partially depleted the pool of calcium releasable by tBOOH. However, exposure to tBOOH did not affect calcium release following treatment with 1799 or KCN (data not shown). This phenomenon was described previously by Bellomo et al. (22) and was attributed to a mitochondrial sensitive and insensitive pool releasable by hydroperoxides. Fig. 5A depicts the translocation of annexin VI following exposure to the mitochondrial uncoupler 1799 and the mitochondrial inhibitor KCN. Both exposures resulted in the translocation of annexin VI from the membrane equivalent to amounts observed following treatment with 100 \(\mu M\) tBOOH. This suggested dependence upon not only lipid and calcium for binding of annexin VI to the membrane, but also ATP. To confirm this, measurements of intracellular ATP concentration were made. Fig. 5B depicts the effect of the mitochondrial uncoupler 1799 and hydroperoxides upon alveolar macrophage intracellular ATP concentration. While uncoupling the mitochondria with 1799 produces a dramatic decrease in cellular ATP, there were also significant decreases produced by the exposure to tBOOH. ATP has recently been identified to increase annexin binding to membranes in the presence of calcium \(\text{in vitro}\) (23). Here we have presented a cellular model of the same process, demonstrating a direct correlation between decreased cellular ATP and annexin translocation from the

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**Fig. 2. Translocation of annexin VI.** Alveolar macrophages were treated, lysed, and separated into three fractions: 20,000 \(\times\) g pellet (the crude particulate); 105,000 \(\times\) g pellet (membrane); and C (cytosol). The control was untreated macrophages in lysis buffer containing 1 mM CaCl\(_2\). Pretreatment with tBOOH promotes the translocation of the protein. This blot is representative of three separate experiments and depicts only the 68-kDa band. The band was compared with a fibroblast control (supplied with the antibody), and disappeared with preabsorption of the antibody. Densitometry represents the percentage of each sample in each cell fraction. Substitution of a lysis buffer without Ca\(^{2+}\) but containing 1 mM EGTA just prior to sonication produced a small degree of translocation, mostly to the 20,000 \(\times\) g pellet.

**Fig. 3. Increase in cytosolic annexin VI as a function of tBOOH concentration.** Annexin VI increases in the cytosol upon exposure to 25 \(\mu M\) tBOOH and is maximized at 100 \(\mu M\) tBOOH. Treatment with lethal levels of hydroperoxide (1 mM) does not increase annexin VI translocation into the cytosol. This blot is representative of three separate experiments. Densitometry represents the percent cytosol control.

**Fig. 4. Increase in cytosolic annexin VI as a function of time.** Further investigation into the translocation of annexin VI following exposure to hydroperoxide shows that translocation increases not only as a function of concentration, but is also dependent upon the time of hydroperoxide exposure. This suggests that the increase and decrease in intracellular calcium during exposure to hydroperoxides is occurring through at least two different mechanisms. This blot is representative of three separate experiments. Densitometry represents percentage cytosol control.
damage to the plasma membrane Ca\(^{2+}\) dants (24–27). We suspected that thiol oxidation resulted in
mained inoperative. Treatment with 200 \(\mu\)M tBOOH (Fig. 6
also observed for treatment with 200 \(\mu\)M tBOOH (Fig. 6
B)
vented the accumulation of \([Ca^{2+}]\) which inhibit or uncouple the mitochondria to the partial de-
membrane. The decrease in ATP also links the effect of agents
which inhibit or uncouple the mitochiondria to the partial de-
plication of increases in \([Ca^{2+}]\), stimulated by hydperoxide exposure. However, since not all of the tBOOH pool is releas-
able by mitochondrial uncouplers or inhibitors, it also implies mechanisms other than ATP depletion for annexin VI translation
during exposure to hydperoxide. Other potential mechanisms underlying the translation of annexin VI are oxidative modification of membrane lipids and or integral proteins.

**Transient Nature of Increased [Ca\(^{2+}\)]\(_i\) following Exposure to tBOOH** —The translocation pattern of annexin VI following exposure to hydperoxide predicts that there would be an increase in the concentration of free calcium at the membrane throughout the metabolism of the hydperoxide. However, we have observed that the increase in \([Ca^{2+}]\), is transient at concentrations <50 \(\mu\)M, and sustained at concentrations >50 \(\mu\)M (2, 10). Inhibition of the plasma membrane or the endoplasmic reticulum Ca\(^{2+}\)-ATPase is common during exposure to oxidants (24–27). We suspected that thiol oxidation resulted in damage to the plasma membrane Ca\(^{2+}\) pump resulting in re-
storible inhibition of the Ca\(^{2+}\) pump at the lower concentrations, whereas at higher concentrations the Ca\(^{2+}\) pump re-
mained inoperative. Treatment with 200 \(\mu\)M DTT completely restored \([Ca^{2+}]\), to base-line concentrations during exposure to tBOOH (Fig. 6A). Pretreatment with 200 \(\mu\)M DTT also pre-
vented the accumulation of \([Ca^{2+}]\), in response to tBOOH. Incubating the cells in the absence of extracellular calcium and with Indo-1 pentapotassium salt, shows that treatment with 200 \(\mu\)M DTT results in the immediate extrusion of calcium from the cell during exposure to tBOOH (Fig. 6B). The extrusion was also observed for treatment with 200 \(\mu\)M DTT following exposure to 25 \(\mu\)M tBOOH for 5 min, which then decreased with time following exposure. Likewise, during the recovery phase of \([Ca^{2+}]\), following exposure to 25 \(\mu\)M tBOOH, there is a gradual

**Fig. 5. Increases in cytosolic annexin VI with 1799 and KCN.** A, the translocation of annexin VI following exposure to the mitochondrial uncoupler 1799 and the mitochondrial inhibitor KCN. Alveolar macroph-
ages were exposed to 1799 with oligomycin or KCN which resulted in the translocation of annexin VI from the membrane equivalent to amounts observed following treatment with 100 \(\mu\)M tBOOH. The blot is representative of two separate experiments. B, the effect of the mitochiondrial uncoupler 1799 with oligomycin and hydperoxides upon alveolar macrophage intracellular ATP concentration. The data are expressed as the mean ± S.E. of four separate samples.

**Fig. 6. Restoration of [Ca\(^{2+}\)]\(_i\) and Ca\(^{2+}\) efflux by dithiothreitol.** A, treatment of Indo-1 loaded alveolar macrophages with 200 \(\mu\)M DTT completely restored \([Ca^{2+}]\), to base-line concentrations during exposure to 100 \(\mu\)M tBOOH. B, incubating the macrophages in the absence of extracellular calcium and with Indo-1 pentapotassium salt shows that treatment with 200 \(\mu\)M DTT results in the rapid extrusion of Ca\(^{2+}\) from the cell.

**DISCUSSION**

The annexins are a family of proteins which bind Ca\(^{2+}\) in a phospholipid-dependent manner (15, 28, 29). The family consists of a highly conserved 4- or 8-fold repeat of 70 amino acid residues (30, 31). A variable N-terminal domain is thought to provide functional diversity. Annexin VI is a 68-kDa protein that preferentially binds to the acidic phospholipids resulting in its primary association with the plasma membrane (15, 16). Annexin VI is composed of eight repeats (17, 18) with a binding capacity of up to eight Ca\(^{2+}\) per protein (32, 33). The large binding capacity for Ca\(^{2+}\) and location on the inner membrane has suggested a role for the protein in cell signal transduction. Thus far, annexins have been identified as mediators of cytoskeletal-membrane interactions (34), inhibitors of phospholipase A\(_2\) and protein kinase C activity (35–37), and regulators of exocytosis, endocytosis, and clatherin-coated pit formation (38, 39).

There are three issues to consider with regard to the trans-
location of annexin VI: where it goes, what happens to its
bound Ca\(^{2+}\), and what causes the release into the cytosol dur-
ing oxidative stress. Annexin attaches to both the plasma mem-
brane, partially through Ca\(^{2+}\) and phospholipid interactions,
and to the cytoskeleton (the 20,000 × g fraction) through non-Ca$^{2+}$-dependent interaction (34). When control cells were disrupted, the annexin remained primarily with the plasma membrane. With EGTA in the lysis buffer, the plasma membrane interaction was partially disrupted and significantly more of the annexin went with the cytoskeleton during fractionation compared with control cells. Annexin VI binding to the plasma membrane may be largely Ca$^{2+}$-independent as suggested by the observation that 70% of the protein remained with the membrane fraction even in the presence of EGTA. Nevertheless, even though Ca$^{2+}$ is not required for binding to the membrane, annexin VI will still bind Ca$^{2+}$ in a phospholipid-dependent manner.

Following treatment with tBOOH, annexin VI increased in the cytosol but not in the 20,000 × g fraction. Although binding of annexin VI may not be completely Ca$^{2+}$-dependent, any annexin VI released by tBOOH would have released most of its bound Ca$^{2+}$ as it dissociated from the membrane phospholipid. Indeed, the movement of annexin VI to the cytosol correlated with a tBOOH dose- and time-related increase in free Ca$^{2+}$, which occurred near the plasma membrane (Fig. 1).

Monocytes have previously been shown to contain 50–100 μg annexin VI/10$^5$ cells (36). Potentially, if all the annexin VI dissociated from the membrane, total cytoplasmic Ca$^{2+}$ would increase ~0.75–1.5 μM assuming similar annexin VI content in monocytes and alveolar macrophages. This does not include the potential contribution of Ca$^{2+}$ release from other annexins that may also dissociate from membrane. However, release of Ca$^{2+}$ from annexin VI alone has a potential far in excess of what occurs during normal signal transduction or sublethal oxidative stress. To account for the 50 μM increase observed with tBOOH (Fig. 6), only 3–7% of the annexin VI need be released. However, with the capacity of mitochondria to take up Ca$^{2+}$, it is not unrealistic to expect as much as 30% (the amount of annexin release apparent in Fig. 2) to be required. Given all the assumptions and estimates, the closeness of this approximation is striking.

It has been observed that the binding affinity of annexin to phospholipid in the presence of Ca$^{2+}$ increases in the presence of physiologic levels of ATP (23). We have observed decreases in cytosolic ATP, which correlated with annexin VI translocation. However, there is no direct evidence that these decreases in ATP were the direct cause of protein-membrane dissociation.

Major cellular targets of oxidative stress include polyunsaturated fatty acids and cellular proteins. Membrane lipids are susceptible to peroxidation resulting in the formation of lipid radicals, which in conjunction with oxygen radicals can attack residues of proteins. Metabolism of lipid hydroperoxides and H$_2$O$_2$ also results in production of oxidized glutathione, which then reacts with protein cysteine residues to form mixed disulfides. These types of alterations have been observed following the stimulation of the respiratory burst, due to exposure of the plasma membrane to H$_2$O$_2$ (40). Thus, these types of disruptions to the plasma membrane, in conjunction with the potential effect of ATP depletion, may result in detachment of annexin VI from the membrane and the inhibition of the membrane Ca$^{2+}$-ATPase. The loss of lipid or protein binding capacity results in a conformational change in annexin VI resulting in only one functional Ca$^{2+}$ binding site, reducing the affinity for calcium binding (16). This confers the release of calcium from the protein upon dissociation from the membrane. The apparent translocation of annexin VI during exposure to tBOOH results in initial increases in free Ca$^{2+}$ near the membrane. The increase in [Ca$^{2+}$]$_i$ was sustained through inactivation of the Ca$^{2+}$-ATPase in the plasma membrane, which has been demonstrated in erythrocytes (24, 27). As shown previously, the inactivation of the Ca$^{2+}$ pump was reversible by addition of thiol-reducing agents (25, 26). Thus, the thiol-reducing agent, DTT, restored the ability of [Ca$^{2+}$], to return to base line.

Despite the difference in the source of free calcium during oxidative stress and receptor-mediated signaling, the hydroperoxide induced changes in [Ca$^{2+}$], can modulate the signal transduction for the respiratory burst, and potentially any other calcium dependent process, especially in the vicinity of the plasma membrane. Furthermore, dissociation of the protein from the membrane would allow activation of phospholipase A$_2$ and, in conjunction with increased [Ca$^{2+}$], alter phagocytosis and chemotaxis in the oxidant exposed macrophage. Thus, we have identified a potential mechanism (Fig. 7) linked to morphological changes observed in macrophages exposed to oxidative stress or prolonged inflammatory reactions.

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