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Mitochondrial Reactive Oxygen Species Regulate Spatial Profile of Proinflammatory Responses in Lung Venular Capillaries

Kaushik Parthasarathi,* Hideo Ichimura,* Sadiqa Quadri,* Andrew Issekutz,‡ and Jahar Bhattacharya‡‡

Cytokine-induced lung expression of the endothelial cell (EC) leukocyte receptor P-selectin initiates leukocyte rolling. To understand the early EC signaling that induces the expression, we conducted real-time digital imaging studies in lung venular capillaries. To compare receptor- vs nonreceptor-mediated effects, we infused capillaries with respectively, TNF-α and arachidonate. At concentrations adjusted to give equipotent increases in the cytosolic Ca²⁺, both agents increased reactive oxygen species (ROS) production and EC P-selectin expression. Blocking the cytosolic Ca²⁺ increases abolished ROS production; blocking ROS production abrogated P-selectin expression. TNF-α, but not arachidonate, released Ca²⁺ from endoplasmic stores and increased mitochondrial Ca²⁺. Furthermore, Ca²⁺ depletion abrogated TNF-α responses partially, but arachidonate responses completely. These differences in Ca²⁺ mobilization by TNF-α and arachidonate were reflected in spatial patterning in the capillary in that the TNF-α effects were localized at branch points, while the arachidonate effects were nonlocalized and extensive. Furthermore, mitochondrial blockers inhibited the TNF-α but not the arachidonate-induced responses. These findings indicate that the different modes of Ca²⁺ mobilization determined the spatial patterning of the proinflammatory response in lung capillaries. Responses to TNF-α revealed that EC mitochondria regulate the proinflammatory process by generating ROS that activate P-selectin expression.


The vast alveolar surface area of the lung potentially constitutes a portal of entry for infected pathogens. Although it is understood that the lung defends against this by mounting an inflammatory response as part of its well-developed innate immunity, it is puzzling as to how the response is spatially patterned in capillaries to maintain optimal conditions for gas exchange. Recruitment of capillary surface area by the inflammatory response could amount to a loss of surface area for gas exchange since the physical presence of inflammatory cells in lung capillaries, or increased capillary leak during the inflammatory response, could impede oxygen transport. The lung’s strategy for avoiding this nonbeneficial competition between defense and gas exchange functions remains unknown.

Critical to the lung’s innate immunity are endothelial cells (EC) that institute rapid signaling by increasing the cytosolic Ca²⁺ concentration. The mitochondrial Ca²⁺ stores (ER) activates Ca²⁺ entry through noncapacitative channels in the cell membrane, or by Ca²⁺ entry through noncapacitative channels activated by direct agonist action (3). Ca²⁺ mobilization also occurs into and out of mitochondria (4, 5), raising the possibility that these organelles may have an impact on patterning the proinflammatory response in the lung capillary.

In this study, we tested the hypothesis that in EC, differences in Ca²⁺ mobilization determine the extent to which mitochondria determine proinflammatory responses in lung venular capillaries. To distinguish between CCE and non-CCE, we exposed the capillaries to the prototypical inflammatory cytokine, TNF-α that causes receptor-mediated Ca²⁺ entry (6), and to the inflammatory product, arachidonate that mobilizes Ca²⁺ entry directly across the cell membrane by nonreceptor mechanisms (3). At equipotent Ca²⁺ entry increases induced by these agents, we addressed spatial profiles of mitochondrial density and mitochondrial Ca²⁺ (Ca²⁺MIT) content in EC of these capillaries. Our findings indicate that mechanisms of Ca²⁺ mobilization play a critical role in patterning the proinflammatory response in the lung capillary.

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An unexpected result was that TNF-α induced P-selectin expression by augmenting mitochondrial reactive oxygen species (ROS) production.

Materials and Methods
Fluorescent probes and other agents
Fluorescent probes fura-2 AM, MitoTracker Green FM (MTG), chloromethyl tetramethyl rosamine (MitoTracker Orange; MTO), rhod 2 AM, and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR). fura-2F AM and 4,5-diaminofluorescein (DAF 2DA) were from TEF Labs (Austin, TX) and Calbiochem (La Jolla, CA), respectively. Mouse anti-rat-selectin mAb RP-2 was a gift from A. C. Issekutz (Department of Microbiology-Immunology, Dalhousie University, Halifax, Canada). Goat anti-rat TNPFR1 mAb E-20 was from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa-Fluor 488-conjugated goat anti-mouse secondary Ab was from Molecular Probes.

Agents human TNF-α and arachidonic acid, the mitochondrial inhibitor rotenone and antimycin, the NO synthase inhibitor G-nitro-L-arginine methyl ester (L-NAME), NO donor s-nitroso acetyl penicilamine (SNAP), p-fluoro methoxy) phenylhydrazone (FCCP), the Ca2+-ATPase inhibitor 2,5-dietf-butyl-hydroquinone (t-BHQ), the ionositol trisphosphate receptor blocker Xestospongin C (XeC) and the antioxidant trolox were from Calbiochem. The Ca2+ chelator BAPTA-AM was from Molecular Probes.

Agents were infused into capillaries in HEPES-buffered vehicle with 4% glucose.

Preparation of the isolated blood perfused rat lungs and the imaging methods
Lung preparation
Preparation of isolated rat lungs was performed as described previously (2, 7). Briefly, lungs were excised from adult male Sprague Dawley rats and continuously pump-perfused at 14 ml/min with autologous rat blood warmed to 37 °C. The lungs were constantly ventilated at an airway pressure of 5 cm H2O. The pulmonary arterial and left atrial pressures were maintained at 10 and 5 cm H2O, respectively. The lungs were positioned on a vibration-free air table. The lung surface was kept moist with saline warmed to 37 °C.

Substance delivery and concentration
A PE10 (BD Biosciences, Sparks, MD) microcatheter was introduced through the left atrial canula and wedged into the lung microcircuitry. Capillary cell-free conditions were established by flushing with HEPES-buffered Ringer’s solution. The agents were infused at the following concentrations: rhod 2 AM 5 μM, MTG FM 1 μM, MTO 2.5 μM, fura 2 AM 10 μM, DCFH-DA 2.5 μM, DAF 2DA 2 μM, TNF-α 40–200 ng/ml, arachidonate 2–10 μM, saponin 0.01%, antimycin 1 μg/ml, FCCP 400 nM–1 μM, oligomycin 2.5 μg/ml, rotenone 1 μM, BAPTA AM 40 μM, t-BHQ 15 μM, XeC 20 μM, trolox 2 mM, L-NAME 10 μM, P1TO 100 μM, and SNAP 500 μM. FCCP was always infused with oligomycin, to prevent ATP depletion by the mitochondrial ATPase (8). To establish Ca2+-free conditions, capillaries were flushed for 10 min with Ca2+-free HEPES-buffered Ringer’s containing 0.5 mM EGTA.

Although the only cell type in these capillaries are EC (7), fluorophores may leak across the capillary wall to enter cells such as epithelial cells of adjacent alveoli. Fluorophores may also enter cells in the bloodstream such as leukocytes and platelets. We protected against these potential artifacts by maintaining absorptive conditions within the capillary (9) and by clearing the capillaries of blood before dye infusion.

Fluorescence microscopy
Fluorophores were excited using mercury arc lamp illumination directed through appropriate interference filters (XB58/25R and XB62/25R; Omega Optical, Brattleboro, VT) and filter sets (71000, 41001, and 41004; Chroma Technology, Brattleboro, VT). Fluorophore exposures were controlled by a filter wheel (LAMBDAL02-2, Sutter Instrument, Novato, CA). The fluorescence emission was collected using an objective lens (ULMPlanFL ×00/0.8W; Olympus Optical, Melville, NY), passed through an image intensifier (Midnight Sun; Imaging Research, St. Catharine, ON). Images were then recorded and subject to image analysis (MCID5.0; Imaging Research).

Confocal microscopy
Confocal images were obtained using a LSM5-Pascal (Zeiss, Thornwood, NY) confocal imaging system attached to an Axiohot (Zeiss) microscope. Fluorophores were excited using a He-Ne Laser (545 nm) and an Argon laser (492 nm). Emitted fluorescence was collected using objective lenses (Achromplan ×40/0.8W. Achromplan ×63/1.2W; Zeiss). The images were recorded and analyzed using Pascal (Zeiss).

Capillary imaging and analysis
To detect Ca2+-MIT, we infused rhod 2 AM into capillaries for 20 min followed by a Ringer’s flush for 30 min. A capillary was excited at 545 nm and the fluorescence emissions recorded at 560 nm. Capillary Ca2+ was determined as described previously (7). Briefly, capillaries were loaded with fura 2 AM for 30 min and then excited at 340 and 380 nm. The fluorescence emissions at 510 nm were recorded and Ca2+ calculated from a computer-generated 340:380 emissions ratio. ER Ca2+ was determined by fura 2F using a protocol similar to that used for fura 2. To detect EC ROS and NO production, DCFH-DA and DAF 2DA, respectively, were continuously infused into capillaries. Image acquisitions were started 30 min after the start of fluorophore infusion. Capillaries were excited at 490 nm and the emission at 510 nm was recorded. Changes in capillary fluorescence were quantitated in a 4-μm2 area along the capillary wall.

FIGURE 1. Real-time confocal microscopy of EC mitochondria in lung venular capillaries. High power view of an EC (A) showing clumped (arrow) fluorescence of MTO. Dashed line indicates vessel margin. Images of a capillary show fluorescence of MTG (B) and rhod 2 (C) separately and superimposed (D). Arrow shows blood flow direction. Branch point (white arrowhead) and midsegmental locations (yellow arrowhead) are indicated. Replicated four times. E, Indicated fluorescence quantifications were obtained in a 4-μm2 window placed over capillary images. Mean ± SE; n = 4 experiments. *p < 0.05 compared with branch point.
In situ indirect immunofluorescence

To determine EC P-selectin expression, we infused RP-2 (3.5 μg/ml) into the capillary for 3 min, followed by the secondary AlexaFluor-488-conjugated Ab (2 μg/ml) for 2 min. Unbound fluorescence was removed by flushing for 1 min with Ringer’s solution. Residual capillary fluorescence was recorded and changes in global fluorescence were reported as changes in P-selectin expression.

Statistics

All data are reported as mean ± SE. Paired observations were compared using paired Student’s *t* test and grouped differences were compared with ANOVA (Newman-Keuls test).

Results

EC mitochondria

To determine mitochondrial density in lung EC in situ, we infused venular capillaries with the mitochondria-sensitive dyes, MTG, MTO, and rhod 2. MTG and MTO localize to mitochondria by binding to the inner membrane (10), while rhod 2, which is Ca2+-sensing, distributes by charge to the anionic mitochondrial matrix (8). Real-time confocal microscopy at high magnification revealed EC mitochondria as clumped fluorescence aggregates of diameter 0.5–1 μm that were organized along the cell periphery (Fig. 1A) (10, 11). Viewed at lower magnification, the dyes appeared colocalized in the capillary wall and were most dominantly fluorescent in EC located at capillary branch points (Fig. 1, B–D). Quantified over equal lengths of vessel wall, MTG fluorescence was more than two times higher at branch points than at midsegments (Fig. 1E).

Rhod 2

To determine the extent of cytosolic uptake, we compared rhod 2 fluorescence against that of the Ca2+-sensitive indicator, fura 2. The membrane-permeabilizing agent saponin (2) transiently increased the fura 340:380 ratio (Fig. 2A) as well as rhod 2 fluorescence (Fig. 2B), indicating that permeabilization increased Ca2+ influx into the cytosol. Subsequently, while the 340:380 ratio decreased to zero, rhod 2 fluorescence returned to baseline and remained steady, indicating that although membrane permeabilization eliminated fura 2 that localized to the cytosol, rhod 2 was not eliminated because it was compartmentalized to mitochondria and not the cytosol. Baseline fluorescence for each fluorophore was stable for up to 40 min (Fig. 2, C and D). To inhibit Ca2+MIT uptake, we infused FCCP that uncouples oxidative phosphorylation (4, 8, 12). FCCP decreased rhod 2 fluorescence, while causing a small increase in Ca2+CYT (Fig. 2, E and F). These responses indicate that with inhibition of Ca2+ uptake in mitochondria Ca2+MIT export predominated and accounted for a small Ca2+ increase in the cytosol. These responses affirmed that rhod 2 fluorescence reflects Ca2+MIT dynamics (4, 8).

EC Ca2+

TNF-α increased Ca2+CYT and Ca2+MIT in a concentration-dependent manner (Fig. 3, A–C). FCCP blocked the Ca2+MIT but not the Ca2+CYT response (Fig. 3D). However, rotenone that is not expected to affect mitochondrial or Ca2+CYT in the short term had no effect on either (Fig. 3D). Arachidonate also caused concentration-dependent Ca2+CYT increases, but in contrast to TNF-α, it did not increase Ca2+MIT (Fig. 4). Not shown are our determinations in which we confirmed the absence of pericapillary edema formation that is evident as interstitial widening and loss of image quality, or alterations in blood flow following infusions of TNF-α and arachidonate. Hence, neither agent caused detectable capillary damage.

To characterize these Ca2+ responses further, we considered CCE mechanisms. Two agents that inhibit Ca2+ release from ER, namely XeC that inhibits InsP3 receptors (13) and t-BHQ that depletes the stores by blocking ER Ca2+-ATPase (12, 14), inhibited the TNF-α-induced Ca2+MIT and Ca2+CYT responses (Fig. 5A). However, neither agent blocked the arachidonate-induced Ca2+CYT increase (Fig. 5B). Furthermore, capillary infusions of Ca2+-depleted buffer blocked the Ca2+CYT increase to TNF-α by 40% (*p* < 0.05), while that to arachidonate was blocked completely (Fig. 5C). In affirmation of previous reports (6, 15), these findings indicate that the TNF-α-induced Ca2+CYT increases resulted from CCE, while the arachidonate-induced ones resulted entirely from non-CCE (5).

Because pharmacological inhibitors may have nonspecific effects, we determined store release by quantifying the fluorescence of the cell permeable dye fura 2FF, a low Ca2+ affinity derivative of fura 2 that localizes to the Ca2+-rich ER compartment (16). At baseline, fura 2FF fluorescence was spatially nonuniform being most prominent at branch points and almost nonexistent at midsegmental locations (Fig. 6A), indicating that EC containing high density of Ca2+ stores were distributed nonuniformly in the capillary. The fluorescence was steady for up to 30 min under baseline conditions. However, the Ca2+-ATPase inhibitor t-BHQ that depletes ER Ca2+ induced the expected reduction of fura 2FF fluorescence under Ca2+-free conditions (Fig. 6, B and C). The fluorescence was unaffected by FCCP (data not shown), thereby ruling
out mitochondria as a source of the fluorescence. These results affirmed fura 2FF fluorescence as reflecting ER Ca\textsuperscript{2+} levels (16).

TNF-\alpha decreased fura 2FF fluorescence, but arachidonate had no effect (Fig. 6, B and C). The TNF-\alpha-induced response was abolished in the presence of both t-BHQ and XeC (Fig. 6D). However, FCCP did not affect these responses (Fig. 6D). These findings indicate that TNF-\alpha, but not arachidonate, caused store depletion of Ca\textsuperscript{2+} that was mediated by InsP3 and that was independent of Ca\textsuperscript{2+} \textsubscript{MIT} uptake.

**EC ROS**

To determine whether these Ca\textsuperscript{2+} responses induced EC ROS production, we infused DCFH-DA that intracellularly de-esterifies mAb, RP2 (Fig. 7A). Hence, these responses were not attributable to ROS production. After washing out trolox, an infusion of H\textsubscript{2}O\textsubscript{2} elicited increase of DCF fluorescence (data not shown), indicating that the inhibitory responses to trolox were not attributable to DCF quenching, or to DCF leakage from mitochondria in the arachidonate effect, but also the possibility that its inhibitory effects resulted from blockade of these Ca\textsuperscript{2+} \textsubscript{Cyt} responses (Fig. 8B).

The mitochondrial blockers rotenone and FCCP each blocked the DCF fluorescence increase to TNF-\alpha, but not to arachidonate, indicating that the responses were attributable to ROS production. After washing out trolox, an infusion of H\textsubscript{2}O\textsubscript{2} elicited increase of DCF fluorescence (data not shown), indicating that the inhibitory responses to trolox were not attributable to DCF quenching, or to DCF leakage from mitochondria. The inability of the NO scavenger, PTIO (21), also failed to have any effect on TNF-\alpha's DCF response (Fig. 9C). These findings taken together with the complete inhibition of

Dose-dependent responses with respect to baseline. \(*, p < 0.05\) compared with 0 ng/ml. D, Differential inhibitory effects to FCCP (FC; 400 nM), and rotenone (RO; 1 \mu M). Mean ± SE. \(*, p < 0.05\) compared with response without inhibitor.

**FIGURE 4.** Ca\textsuperscript{2+} \textsubscript{Cyt} responses to arachidonate. AA, arachidonate. A, Traces represent changes in Ca\textsuperscript{2+} \textsubscript{Cyt} (top panel) and rhod 2 fluorescence (bottom panel) in response to infusions of AA (10 \mu M). B, Dose responses and the effect of mitochondrial blockers rotenone (RO; 1 \mu M) and FCCP (FC; 1 \mu M) on AA-induced Ca\textsuperscript{2+} \textsubscript{Cyt} responses.
the DCF response by trolox (Fig. 8A) indicate that the increase of DCF fluorescence was attributable entirely to ROS production.

**P-selectin**

Both TNF-α and arachidonate markedly increased P-selectin expression on the EC lining of these capillaries (Fig. 10, A–C). However, the fluorescence increases were patchy for TNF-α, but considerably more uniform for arachidonate. For both agents, trolox blocked the response (Fig. 10D), indicating that ROS determined the P-selectin expression. Rotenone and FCCP blocked the TNF-α-induced P-selectin expression (Fig. 10D), indicating that mitochondrial mechanisms were also responsible for this effect. In contrast, the arachidonate-induced expression was unaffected by mitochondrial blockers (Fig. 10D), indicating first the noninvolvement of mitochondria in this response and second the absence of nonspecific inhibition of P-selectin expression by these blockers. These responses indicated that the TNF-α- but not arachidonate-induced exocytosis of P-selectin occurred through mitochondrial mechanisms.

**Ca²⁺ depletion**

To further characterize Ca²⁺-dependent mechanisms, we blocked Ca²⁺ changes by either chelating Ca²⁺cyt with BAPTA or by infusing a Ca²⁺-depleted buffer. BAPTA completely inhibited both TNF-α- and arachidonate-induced ROS production and P-selectin expression (Fig. 11), indicating that the response was Ca²⁺cyt-dependent for both agents. However, infusion of Ca²⁺-depleted buffer only partially inhibited the TNF-α-induced ROS and P-selectin responses, but completely inhibited the arachidonate-induced responses (Fig. 11), indicating that the latter responses were entirely dependent on entry of external Ca²⁺.

**Discussion**

We determined the sequence of proinflammatory signaling in EC of lung venular capillaries using TNF-α and arachidonate as inflammatory agonists and P-selectin expression as a marker of the proinflammatory response. Both agonists rapidly increased Ca²⁺cyt, while also increasing ROS production and P-selectin expression. For both, inhibiting the Ca²⁺cyt increase by intracellular Ca²⁺ chelation with BAPTA, or by infusion of Ca²⁺-depleted buffer, blocked P-selectin expression. This affirms the widely held view that an increase of Ca²⁺cyt constitutes the critical signaling event proximal to P-selectin expression (reviewed in Ref. 2). However, our new results redefine this thinking. Thus, blocking the Ca²⁺cyt increase blocked the ROS response, and
To our knowledge, this is the first evidence that places \( \text{Ca}^{2+}_{\text{CYT}} \)-induced ROS in the intracellular signaling pathway underlying the expression of P-selectin.

**Reactive oxygen species**

The TNF-\( \alpha \)-induced ROS were of mitochondrial origin, as indicated by several findings. TNF-\( \alpha \) increased \( \text{Ca}^{2+}_{\text{MIT}} \), a known stimulator of mitochondrial ROS production (22). FCCP inhibited both the \( \text{Ca}^{2+}_{\text{MIT}} \) increase as also the induced ROS production, thereby implicating \( \text{Ca}^{2+}_{\text{MIT}} \) directly in the induction of the ROS. Rotenone also inhibited the TNF-\( \alpha \)-induced ROS increase, while antimycin enhanced it. Because these blockers did not inhibit the concomitant \( \text{Ca}^{2+}_{\text{CYT}} \) increases, we may rule out the possibility that the inhibitions were due to nonspecific effects of the blockers. Previous reports have implicated mitochondria (6, 23), NADPH oxidase (24), and cPLA\(_2\) (25) in TNF-\( \alpha \)-induced ROS production in EC. However, in this study the TNF-\( \alpha \)-induced ROS originated exclusively from mitochondria as indicated by the inhibitory effects of the mitochondrial blockers. Rotenone and FCCP both also inhibited P-selectin expression. Hence, for the first time, these findings implicate EC mitochondria as initiators of the proinflammatory response in these capillaries.

We considered the possibility that the increase of DCF fluorescence resulted from diffusion of \( \text{H}_2\text{O}_2 \) into EC from nonspecific extracellular sources. However, infusion of the anti-TNFR1 mAb, E-20 (2), blocked the TNF-\( \alpha \)-induced ROS production, indicating not only that the effect was receptor-mediated, but also that the DCF response was entirely attributable to ROS production in EC. Moreover, catalase that hydrolyzes extracellular \( \text{H}_2\text{O}_2 \) did not block the TNF-\( \alpha \)-induced response, hence \( \text{H}_2\text{O}_2 \) originating from a source external to EC was not a factor in these responses.

Mitochondrial ROS production may be pathologic, since it abolishes the mitochondrial potential, leading to release of cytochrome \( c \) and apoptosis (18, 26). However, to the extent that we could determine, the present levels of ROS were not cytotoxic since we detected neither mitochondrial damage (Fig. 3A), nor damage to the cell membrane that would have been evident in loss of cell fluorescence resulting from leakage of intracellular fluorescent indicators such as fura 2. Furthermore, the responses returned to baseline levels after transient increases to TNF-\( \alpha \) in the poststimulus period and they could be repeated in the same capillary. These considerations indicate that the present TNF-\( \alpha \)-induced mitochondrial ROS production was a constitutive, not a pathologic, feature of cell signaling.

The mechanisms by which ROS induced exocytosis are not clear. One possibility is that diffusible ROS, such as \( \text{H}_2\text{O}_2 \), inhibits cytosolic tyrosine phosphatases (27) to activate tyrosine kinases of the Src family (28) that may regulate exocytosis. Tyrosine kinases are implicated in some forms of secretion as in neutrophils in which secretion is blocked by the broad-spectrum tyrosine kinase inhibitor, genistein, as well as by PP1, an inhibitor of Src family kinases (29). Moreover, secretion is attenuated in mice deficient in Src family kinases (29), and the tyrosine kinase, Syk, plays a pivotal role in high-affinity IgE receptor-induced secretion in mast cells and basophils (30). The extent to which these mechanisms applied to the present findings requires further consideration.

Arachidonate, as different from TNF-\( \alpha \), used nonmitochondrial mechanisms for ROS production. This was evident in that arachidonate had no effect on \( \text{Ca}^{2+}_{\text{MIT}} \) and that mitochondrial blockers failed to inhibit the arachidonate-induced ROS response. Although we did not identify the source, in neutrophils and eosinophils arachidonate-induced ROS production occurs through activation of NADPH oxidase in the plasma membrane (31, 32). It is proposed that influx of external \( \text{Ca}^{2+} \) primes NADPH oxidase (33). Our blocking the ROS response blocked P-selectin expression. These findings, which were common to both agonists, indicate that an increase of \( \text{Ca}^{2+}_{\text{CYT}} \) was not itself sufficient to induce P-selectin expression and that downstream induction of ROS was required.

![FIGURE 7. ROS production in lung capillaries. Capillaries were infused with vehicle (baseline), TNF-\( \alpha \) (TNF; 200 ng/ml), arachidonate (AA; 10 \( \mu \)M), trolox (TR; 2 mM). Mean ± SE. A–C, Images of single capillaries show DCF fluorescence. Intensity code indicates fluorescence levels. Dashed lines indicate vessel margins. Branch point (arrowhead) and midsegmental (arrow) locations are indicated. Note fluorescence increase is uneven for TNF-\( \alpha \), but extensive for arachidonate. Replicated six times. D and E, Traces from single experiments show time courses of increase in DCF fluorescence. Agents were infused for durations indicated (bars) in the absence (D) and presence (E) of mAbs E-20 (10 \( \mu \)g/ml) and RP-2 (3.5 \( \mu \)g/ml). F, Concentration-dependent responses. *, \( p < 0.05 \) against highest concentration. G, ROS production at different capillary locations. *, \( p < 0.05 \) compared with branch-point. n = 5 capillaries.](http://www.jimmunol.org/DownloadedFrom/7083)
findings are consistent with these mechanisms in that depleting external Ca\(^{2+}\) completely inhibited the arachidonate-induced ROS production, supporting the notion that Ca\(^{2+}\) entry is critical for this response.

**Ca\(^{2+}\) mobilization**

At concentrations that induced equipotent Ca\(^{2+}\) CYT increases (Fig. 3A), TNF-\(\alpha\) and arachidonate activated distinctly different mechanisms of Ca\(^{2+}\) mobilization. First, TNF-\(\alpha\) but not arachidonate reduced the fluorescence of fura 2FF. This TNF-\(\alpha\) effect was abolished by blocking ER Ca\(^{2+}\) release, consistent with the notion that TNF-\(\alpha\) causes InsP3-dependent Ca\(^{2+}\) release from ER (6, 15). Second, TNF-\(\alpha\) but not arachidonate increased Ca\(^{2+}\) MIT. These results suggest that ER Ca\(^{2+}\) release was critical for the Ca\(^{2+}\) MIT increase in support of the view that the spatial proximity of ER and mitochondria facilitates Ca\(^{2+}\) mobilization between these compartments (34). Third, depletion of external Ca\(^{2+}\) blocked the Ca\(^{2+}\) CYT response to TNF-\(\alpha\) only partially, but that to arachidonate completely. We interpret from these findings that store depletion followed by CCE accounted for the TNF-\(\alpha\)-induced Ca\(^{2+}\) CYT increases, while those for arachidonate were entirely attributable to influx of external Ca\(^{2+}\).

Mitochondria regulate Ca\(^{2+}\) CYT by both importing and exporting Ca\(^{2+}\) across the inner membrane by means of a uniporter and an mCa\(^{2+}\)/Ca\(^{2+}\) exchanger, respectively (5, 35). In several cell types (35, 36), the net effect is to buffer increases in Ca\(^{2+}\) CYT and thereby, negatively regulate aspects of Ca\(^{2+}\) CYT-dependent cell function, as for example, catecholamine secretion in chromafﬁn cells (36). Mitochondrial buffering is revealed by inhibiting Ca\(^{2+}\)\(_{\text{MIT}}\) uptake that augments induced Ca\(^{2+}\) CYT increases (5, 35, 36). By contrast, in our experiments, blockade of Ca\(^{2+}\)\(_{\text{MIT}}\) uptake by FCCP did not modify concomitant TNF-\(\alpha\)-induced Ca\(^{2+}\) CYT increases, indicating the absence of a detectable mitochondrial buffering effect.

**Spatial patterning**

The ER and mitochondrial distributions were regionally coupled in these capillaries in that fluorescence for both localized dominantly at branch point EC. TNF-\(\alpha\)’s effects being mitochondria-dependent localized to branch point EC. However, arachidonate’s effects being mitochondria-independent were not subject to similar spatial
constraints. Evidently, EC mitochondrial density spatially patterned the capillary’s proinflammatory response to TNF-α. Although both arachidonate and TNF-α induced Ca\(^{2+}\) influx, nonmitochondrial ROS were activated by external Ca\(^{2+}\) entry attributable to arachidonate, but not by the influx resulting from TNF-α-induced CCE. This differential response indicates that signaling pathways to ROS production are partitioned by not just an increase of Ca\(^{2+}\)\textsubscript{cyt}, but by the mechanism of Ca\(^{2+}\) mobilization. Two classes of Ca\(^{2+}\)-dependent agonists may exist, namely those that recruit ER-mitochondrial mechanisms, and those that favor direct Ca\(^{2+}\) entry, as exemplified in this study by TNF-α and arachidonate, respectively. However, the extent to which these separate strategies of Ca\(^{2+}\) mobilization, hence of spatial patterning, apply to other inflammatory agonists requires further consideration.

**FIGURE 10.** P-selectin expression in lung capillaries. A–C, Images show residual fluorescence of a capillary infused with a P-selectin Ab (RP-2; 3.5 μg/ml) followed by an Alexa Fluor-488-conjugated secondary Ab (2 μg/ml). Panels show capillary fluorescence in response to infusions of vehicle (A), TNF-α (TNF; 200 ng/ml) (B), and arachidonate (AA; 10 μM) (C). Intensity code indicates gray levels. Dashed lines indicate capillary margins. Branch point (arrowhead) and midsegmental (arrow) capillary locations are indicated. Replicated seven times. D, Responses are fluorescence increases above that of vehicle-infused capillaries (baseline). Agonist responses were paired. TNF, 200 ng/ml; AA, 10 μM; rotenone (RO; 1 μM); FCCP (FC; 1 μM); trolox (TR; 2 mM); infusion of secondary Ab (2γ; 2 μg/ml) alone. *, p < 0.05 compared with responses without inhibitors. Mean ± SE. n = number of experiments.

**FIGURE 11.** Effects of inhibiting Ca\(^{2+}\) mobilization in EC of lung capillaries. Effects of TNF-α (TNF; 200 ng/ml) and arachidonate (AA; 10 μM) on ROS (A) and P-selectin expression (B) in the presence of infusions of the Ca\(^{2+}\)-chelator BAPTA (BA; 40 μM) and of Ca\(^{2+}\)-depleted Ringer’s solution containing 0.5 mM EGTA (Ca\(^{2+}\)-). Mean ± SE. *, p < 0.05 compared with responses without inhibitors. n = number of capillaries.

**P-selectin**

Our finding that TNF-α causes P-selectin expression in lung venular capillaries is similar to the TNF-α response in systemic venules (37). In addition, TNF-α increases P-selectin expression in human pulmonary microvascular EC (38) and in HUVEC (39), but not in human pulmonary arterial or dermal EC (38). Our findings together with these reports, indicate that in lung, TNF-α-induced P-selectin expression may be restricted to microvascular EC (38) and that the expression may be an early event that is not detectable after several hours of TNF-α exposure (40). In EC derived from large vessels, TNF-α exposure for several hours causes expression of E-selectin and ICAM-1 by inducing ROS-mediated increase in gene expression of these molecules (41, 42). Taking these findings together with ours, we suggest that TNF-α-induced ROS enable EC expressions of different leukocyte adhesion receptors in a time-dependent manner, such that P-selectin exocytosis occurs immediately, while E-selectin and ICAM-1 expressions are induced at later time points.

In conclusion, our findings with TNF-α reveal the new insight that in EC, mitochondrial ROS may act as diffusible messengers to induce proinflammatory signaling. Previously, mitochondrial ROS have been implicated in signaling related to gene transcription (43), hypoxic pulmonary vasoconstriction (44), insulin secretion (45), and platelet aggregation (46). The present branch point dominant proinflammatory response to TNF-α may reflect a mechanism that protects capillary midsegments from deleterious inflammatory effects that could interfere with gas exchange and reduce blood flow. By contrast, the spatially extensive effects of arachidonate may exemplify the pattern of a more fulminant response. These considerations may be relevant to understanding the extent to which the spatial profile of vascular inflammation determines lung injury.

**References**


