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IN VITRO MODULATION OF HUMAN NEUTROPHIL SUPEROXIDE ANION GENERATION BY VARIOUS CALCIUM CHANNEL ANTAGONISTS USED IN ISCHEMIA–REPERFUSION RESUSCITATION

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Abstract—Generation of toxic oxygen species by activated polymorphonuclear leukocytes (PMNs) may represent an important mechanism of ischemia–reperfusion injury. Concentration–response data concerning inhibition of superoxide anion ($O_2^{\cdot}$) generation by NADPH oxidoreductase (NADPH OR) from isolated human PMN were generated for five calcium channel antagonists commonly utilized in ischemia–reperfusion investigational therapeutics. Regression analysis derived IC_{50} values for verapamil, nimodipine, nicardipine and lidoflazine were 45, 20, 12 and 7 μM respectively. Inhibition of the extent of reaction at 5 min paralleled inhibition of initial velocity. No inhibition by flunarizine was noted at concentrations ≤ 25 μM (where it did not alter reaction mixture composition). Only nicardipine demonstrated a significant concentration–response effect relative to prolonging lag time preceding $O_2^{\cdot}$ synthesis. Inhibition appeared at least partially reversible for all five agents. Neither PMN activation/desensitization, free-radical scavenging, nor PMN cytotoxicity appeared to be involved in the inhibition of PMN $O_2^{\cdot}$ synthesis by these agents. Ca^{2+} antagonist inhibition of PMN NADPH OR appears to involve more than simple inhibition of Ca^{2+} flux across the PMN plasma membrane. Direct inhibition of the intracellular events involved in the activation and/or activity of NADPH OR may be operative.

Ischemia–reperfusion pathology underlies a significant cause of morbidity and mortality for critically ill patients [1]. Myocardial infarction, cerebral ischemic stroke, circulatory shock, cardiopulmonary bypass and organ transplantation represent important examples of this phenomenon [2]. Oxyradicals are abundantly produced in reperfused ischemic tissues and may contribute to resultant endothelial damage. Sources of the toxic oxygen species may be numerous, including polymorphonuclear leukocytes (PMNs) which are known to accumulate in post-ischemic tissue (vascular endothelium and parenchyma) within minutes to hours after establishment of reflow [e.g. Refs 3–5]. Stimulated PMNs may release active oxygen species (as well as a variety of hydrolases) into the surrounding milieu resulting in host autoinjury. Neutrophil NADPH oxidoreductase (NADPH OR), a plasmalemma bound flavoprotein/cytochrome complex, is responsible for synthesis of $O_2^{\cdot}$ as the paramount event in the PMN respiratory burst [6]. Additional toxic oxygen species (H_2O_2, $HO^{\cdot}$, HO^-, chloroamines) are derived from $O_2^{\cdot}$ by enzymatic and transition metal catalysis [7].

Calcium ion flux across the plasmalemma and within the cell [e.g. Ref. 8] is required for activation of the PMN respiratory burst and NADPH OR in particular by most stimuli. Moreover, NADPH OR activity obtained in particulate fractions of PMN lysates itself requires Ca^{2+} and/or Mg^{2+} for full expression of catalytic activity [e.g. Ref. 9]. Calcium channel blocking agents have been investigated extensively for ischemia–reperfusion resuscitation. Such agents have been postulated to have beneficial effects on a variety of potentially deleterious metabolic events including inhibition of neutrophil aggregation and activation. Accordingly, we examined the effects of five calcium channel antagonists, flunarizine (FLU), lidoflazine (LID), nicardipine (NIC), nimodipine (NIM) and verapamil (VER), on one aspect of PMN activation, namely $O_2^{\cdot}$ production, as assessed by their inhibition of PMN NADPH OR ($O_2^{\cdot}$ synthetase) enzyme activity.

MATERIALS AND METHODS

Materials. Lidoflazine, flunarizine and corresponding placebos were supplied by Andrea J. Small of Janssen Pharmaceutica (Piscataway, NJ). Nimodipine (BAY e 9736) and placebo were supplied by Dr Alexander Scriabine of Miles Pharmaceuticals (New Haven, CT). Nicardipine (YC-93) was supplied by Dr Takeo Tanishima from the Japanese Pharmaceutical Co. (Tokyo, Japan). Verapamil (Isoptin®) was supplied by Knoll Pharmaceuticals (Whippany, NJ).

NADPH, NAD, cytochrome *c* (type III), *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), cytochalasin B (CB), superoxide dismutase (SOD), xanthine (X), xanthine oxidase (XO), ethyleneglycolbis(amino - ethylether)tetra - acetate (EGTA), and Ficoll 400 were purchased from the Sigma Chemical Co. (St Louis, MO); 90% Hypaque-M was obtained from Winthrop Laboratories (New York, NY), and Hanks' balanced salt solutions

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(HBSS) from Gibco Laboratories (Grand Island, NY). All other chemicals were of reagent grade and were utilized without additional purification.

PMN isolation. Purified neutrophils were obtained from healthy young adults by differential centrifugation of heparinized (10 units/ml) venous blood. Initial separation of granulocytes from mononuclear cells was achieved utilizing a modification of the Ficoll–Hypaque method with a Ficoll concentration of 8.2% and a solution density of 1.112 to 1.114 [10]. Contaminating erythrocytes were eliminated by water hypotonic lysis followed by 0.6 M KCl rescue. Subsequent washes employed incomplete (without Ca^{2+} and Mg^{2+}) Hanks' balanced salt solution ($HBSS^{--}$) to minimize cell aggregation, while final suspension of the cells was performed in complete HBSS ($HBSS^{++}$). Qualitative analysis of cell preparations by light microscopy after modified Wright's staining routinely demonstrated ≥ 95% PMNs. Quantitation of cells was made using electrical impedance counting with a Hemo-W Coulter Counter (Coulter Electronics, Hialeah, FL). Typical PMN yield was 2–3 × 10^6 PMN per ml of whole blood. For each experiment, PMNs from a single donor were prepared fresh, and utilized within 8 hr of isolation.

Enzyme assays. NADPH oxidoreductase (NADPH OR, $O_2^{\cdot-}$ synthetase) activity was assayed in isolated intact PMNs in microcuvettes with a reaction volume of 1 ml [11]. Assay constituents included NADPH (100 μM), cytochrome *c* (100 μM), CB (10 μM), FMLP (1 μM), ± calcium channel antagonist and PMNs (5 × 10^5) in complete HBSS (Ca^{2+} = 1.26 mM, Mg^{2+} = 0.90 mM), pH 7.4, as the reaction vehicle. Control assays additionally included SOD (100 units/ml). After incubation of the reaction mixture for 5 min at 37°, the respiratory burst was activated by addition of the FMLP. $O_2^{\cdot-}$ production at 37° was followed as the reduction of cytochrome at 550 nm, utilizing an extinction coefficient of 18.5 × 10^3 l/mol/cm. Assays were conducted in a Beckman DU-50 Spectrophotometer equipped with Peltier thermostat, dot matrix printer, and kinetics software (Beckman Instruments, Inc., Irvine, CA). Lag time (seconds from addition of FMLP to linear rate, lag), initial velocity (namomoles $O_2^{\cdot-}$ per minute per 10^6 PMNs, V_i), linearity (seconds of linear initial velocity, lin), and reaction extent (total nanomoles of $O_2^{\cdot-}$ produced by 5 min per 10^6 PMNs) were examined simultaneously. Characteristics of the enzyme assay system [11] are depicted in Fig. 1. After addition of the respiratory burst stimulus (here FMLP), a 20- to 30-sec lag period was typically noted after which maximal initial velocity $O_2^{\cdot-}$ production (V_i) proceeded linearly for about 1 min. Thereafter, a gradual reduction in the reaction rate was noted, with most of the reaction complete by 5 min. Reagent blank assays containing SOD illustrate that nearly all cytochrome *c* reduction occurred as a result of $O_2^{\cdot-}$ production, since with added SOD, $O_2^{\cdot-}$ was quickly dismutated to H_2O_2 and was unavailable for cytochrome *c* reduction. Because of significant quotidian and individual variations in PMN respiratory burst activities, normalization of data for individual experiments to corresponding concurrent control activities was utilized in order to compare

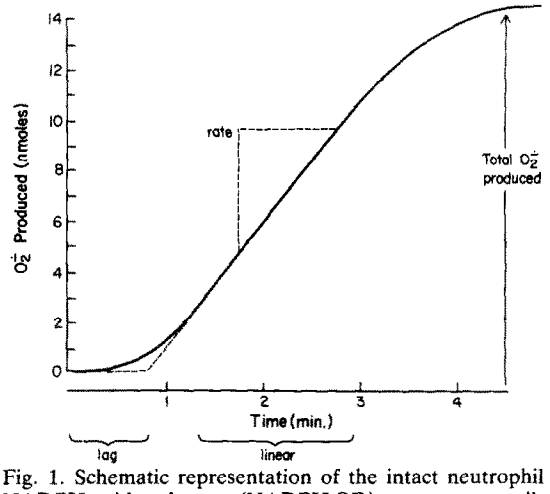

Fig. 1. Schematic representation of the intact neutrophil NADPH oxidoreductase (NADPH OR) assay system utilized in this study, demonstrating graphically definitions of lag time, linearity, V_i, and extent (total $O_2^{\cdot-}$ produced) of reaction.

groups of experiments. Concentration–response data were generated by adding various concentrations of calcium antagonists to the reaction mixture and noting the effect on lag, lin, V_i and extent of reaction relative to control assays without drug.

After isolation, neutrophils were stored gently agitated at high concentration (15–25 × 10^6 PMNs/ml) at 4° on a rocker platform to maximally extend functional activity. Control assays (without drug) were performed intermittently throughout the experiments. In the few cases of prolonged experimental time frame where enzyme activity decay (<20%) was demonstrated, results were corrected to rectify this spontaneous loss of activity. Similarly, at the highest concentration of some drugs tested, drug vehicle was found to be deleterious to PMN NADPH OR activity. Accordingly, concentration–response curves were also constructed for some drug vehicles, so that vehicle effect could be differentiated from actual drug effect.

Ability of calcium antagonist drugs to themselves induce $O_2^{\cdot-}$ synthesis in intact PMNs was evaluated with the above outlined assay system. However, FMLP was omitted and instead various drugs (final concentration = 1.5 IC_{50}) were utilized to attempt to initiate the reaction.

Lactate dehydrogenase activity (LDH) (liberated from traumatized PMNs) was assayed spectrophotometrically by following the reduction of NAD at 340 nm [12].

Additional drug effects. To investigate if calcium antagonist drugs exhibited any direct cytotoxic effects towards PMNs and if their inhibition was reversible, aliquots of PMNs (10 × 10^6) were incubated at 37° for 10 min in complete HBSS with each drug at a concentration of 1.5 IC_{50}. Subsequently, supernatant fractions were separated from the cells by centrifugation (400 *g*, 10 min, 4°). PMNs (in the pellet) were gently resuspended in complete HBSS, quantitated, and reassayed for inducible $O_2^{\cdot-}$ production. Reversibility for each drug was normalized

as a percentage of activity relative to concurrent control cells, exposed to only complete HBSS.

Supernatant fractions were assayed directly for LDH activity. Cytotoxicity was expressed as a percentage of LDH activity released relative to supernatant fraction obtained from completely disrupted PMNs (by sonication, Cole Parmer Ultrasonic Processor, Chicago, IL, at 20 W, 10 sec, twice in an ice–H_2O bath). Similarly, cell counts of the resuspended PMNs were utilized as a gross index of drug-induced cytotoxicity, by relating percentage of intact cells relative to control cells, exposed only to complete HBSS.

To assess if any of the drugs functioned as a free-radical scavenger, a cell-free $O_2^{\cdot}$-generating system was assembled to mimic conditions of the NADPH OR assay. CB (10 μM), cytochrome *c* (100 μM), NADPH (100 μM), XO (0.01 units), and X (50 μM) comprised the basal $O_2^{\cdot}$-generating system [13]. Velocity of $O_2^{\cdot}$ production was followed spectrophotometrically at 550 nm and 37° as ferric cytochrome *c* reduction as outlined above. Individual drugs were added to this reaction mixture at a concentration of 1.5 IC_{50}, and reaction velocity was examined for quenching of cytochrome *c* reduction. Inhibition by SOD (100 units/ml) served as a reference.

To examine the effect of extracellular calcium and magnesium concentration on $O_2^{\cdot}$ production by FMLP-stimulated PMNs, reactions were conducted in complete and incomplete HBSS, as well as in the presence of 5 mM EGTA.

Data analysis. At least three reactions and one SOD control were analyzed for each drug concentration examined. Because of well recognized daily and individual variation in NADPH OR activity [11], drug inhibition was normalized as a percent of concurrent control activity (no drug). At least three separate experiments were performed for each drug. All data are expressed as mean ± standard deviation. The IC_{50} values (inhibitor concentration at which 50% inhibition occurs) were derived from best fit concentration–response regression equations utilizing a Hewlett–Packard System 45 statistical program. To estimate IC_{50}, regression equations were solved for x (drug concentration, μM) with y (% control activity) equal to 50%. Classical techniques to derive a true K_i utilizing double-reciprocal plots ($1/V$ vs $1/S$) are not appropriate in this whole neutrophil system where the concentration of substrates (e.g. intracellular NADPH) cannot be accurately controlled. Although at least three separate concentration–response experiments were conducted for each drug, concentration–response data points within a given experiment are not independent variables. Hence, valid statistical methodology does not exist to describe some type of variance for the regression-derived IC_{50} values. Accordingly, these values must be considered descriptive and not subject to intra-group comparison. Statistical comparisons of various absolute enzyme reaction rates were made utilizing the *t*-test with Bonferroni's correction for multiple comparisons. Significant P values were considered to be $\leq 0.05 \div$ number of multiple comparisons. Significance of lag time prolongation in relation to drug concentration was ascertained using analysis of variance (ANOVA).

Table 1. Effect of nicardipine on prolonging lag time preceding $O_2^{\cdot}$ synthesis by FMLP-activated PMN NADPH oxidoreductase

Nicardipine concentration (μM)	Lag time (sec)
0	19 ± 1
5	22 ± 3
10	22 ± 1
15	28 ± 6
20	46 ± 4
25	52 ± 5
30	54 ± 10

Data presented were taken from a single experiment with three to four assays per nicardipine concentration, but is representative of three separate experiments. Lag times preceding linear production of $O_2^{\cdot}$ are recorded as mean ± SD.

RESULTS

As a reference, average values for the concurrent controls (i.e. no drug) in twenty-four separate experiments included: lag time, 23 ± 6 sec; linearity, 66 ± 18 sec; initial velocity, 6.6 ± 1.6 nmol $O_2^{\cdot}$/min/10^6 PMNs; and extent of reaction at 5 min, 23.3 ± 4.6 nmol $O_2^{\cdot}$/10^6 PMNs. Note that separate concurrent controls (not the mean values recorded above) were utilized for individual experiments. Figure 2 is a composite of the concentration–response data for V_i for VER, NIM, NIC and LID. Computer-generated regression analysis equations and absolute control V_i values are displayed for each drug. Derived IC_{50} values were 45, 20, 12, and 7 μM for VER, NIM, NIC, and LID respectively. At concentrations ≤ 25 μM, FLU had no effect on PMN $O_2^{\cdot}$ production. At higher concentrations, FLU precipitated from solution (augmented buffering capacity vehicle) or drastically reduced reaction pH.

Inhibition of the extent of reaction (total $O_2^{\cdot}$ produced per 10^6 PMNs in 5 min) generally paralleled inhibition of V_i even though the reactions demonstrated non-linearity by 2 min (data not shown). At drug concentrations of 2 IC_{50}, reactions were uniformly complete in less than 3 min for all of the four drugs demonstrating inhibition.

Although it was anticipated that duration of NADPH OR reaction linearity might reflect another aspect of calcium antagonist inhibition of PMN $O_2^{\cdot}$ production, no consistent concentration–response relationship was noted for this variable. Even for cells not exposed to drugs, linearity of the maximum initial velocity was short-lived. This fact underscores the transient nature of the respiratory burst under *in vitro* conditions and emphasizes the need to utilize a continuous initial velocity assay system to accurately examine the kinetics of this reaction.

All drugs demonstrating inhibition appeared to prolong lag time at high concentrations approaching

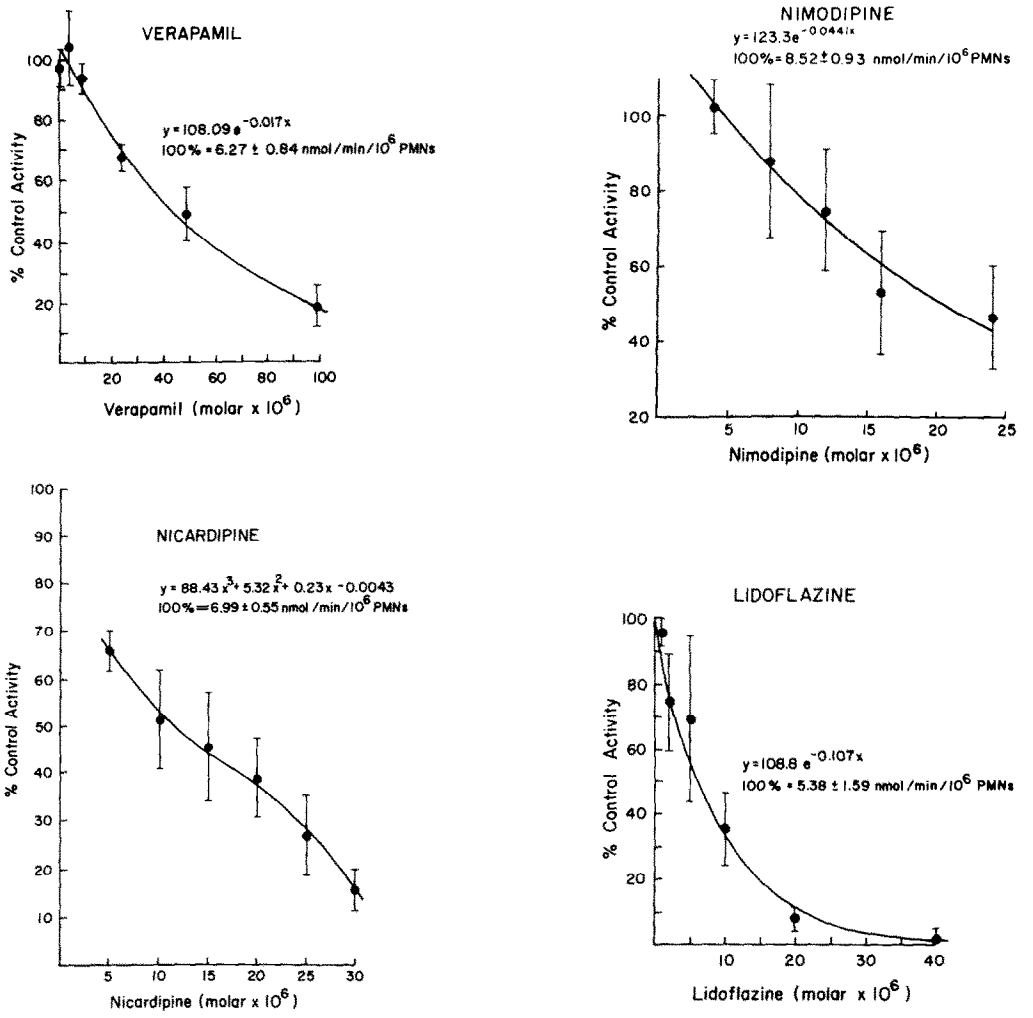

Fig. 2. Concentration–response data indicating the inhibitory effect of verapamil, nimodipine, nicardipine, and lidoflazine on the initial velocity (V_i) kinetics of $O_2^{\cdot}$ production by FMLP-stimulated PMNs. Data are normalized with respect to concurrent control assays containing no drug (i.e. no drug = 100% activity). Absolute mean control activity and the computer-driven regression analysis equation are displayed for each graph, each representing the mean ± SD of three to six experiments.

2 IC_{50}. However, only nicardipine exhibited a consistent type of concentration–response relationship with respect to this parameter ($P < 0.005$ by ANOVA, Table 1).

Inhibition of $O_2^{\cdot}$ synthesis (V_i) by these drugs after a 10-min incubation at 37° with neutrophils was variably reversible. As can be seen in the histogram displayed as Fig. 3, inhibition by VER and NIM was nearly completely reversible. Only cells exposed to NIC and LID demonstrated a statistically significant lack of NADPH OR activity reversibility compared to PMNs exposed only to HBSS (60 ± 7.5 and 35 ± 1.5% respectively; both $P < 0.01$). Similar results were noted for extent of reaction. No consistent irreversible effects on lag time or linearity were noted.

To characterize further the effect of calcium antagonists on PMN $O_2^{\cdot}$ generation, a series of experiments was devised to rule out possible auxiliary effects of these drugs not directly related to inhibition of PMN NADPH OR. Activation/desensitization, cytotoxicity, and free-radical scavenging phenomena were examined separately.

If calcium antagonists possibly caused activation of the PMN respiratory burst (independent of their calcium blocking activities), some of their inhibition of $O_2^{\cdot}$ production by FMLP might be explained on the basis of recognized PMN activation/desensitization phenomenon. That is, prior activation of the cell by a calcium antagonist during the 5-min preassay incubation might render it desensitized to a subsequent stimulation attempt by FMLP. Each drug was tested at a concentration of 1.5 IC_{50} (12.5 μM for FLU) for its ability to activate PMN $O_2^{\cdot}$ synthesis. None were effective in this regard.

Drug-induced neutrophil cytotoxicity would also manifest as depressed $O_2^{\cdot}$ production. This possibility was investigated by incubating PMNs (±FMLP) with calcium antagonists (1.5 IC_{50}, 12.5 μM for FLU), washing out the drug by cen-

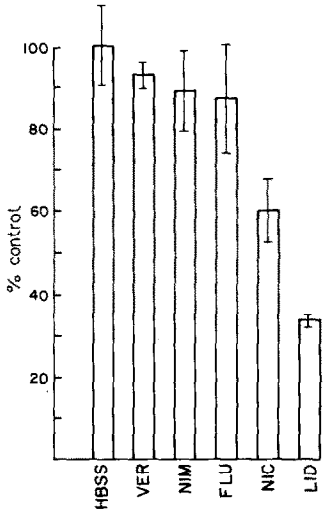

Fig. 3. Reversibility of inhibition of PMN O_2^- synthesis by various calcium antagonists, relative to concurrent control cells not exposed to drugs. Aliquots of 10×10^6 PMNs were incubated with HBSS ± a calcium antagonist at a concentration of 1.5 IC_{50} (12.5 μM for FLU); for 10 min at 37°; washed twice (HBSS) by centrifugation; and then resuspended (complete HBSS), recounted and reassayed for initial velocity production of O_2^- following FMLP stimulation. Each bar represents the mean ± SD of three assays. Absolute specific activities ± SD in nmol O_2^-/min/10^6 PMNs: HBSS, 3.06 ± 0.30; VER, 2.84 ± 0.10; NIM, 2.76 ± 0.34; FLU, 2.67 ± 0.41; NIC, 1.84 ± 0.23; and LID, 1.03 ± 0.045. Only NADPH OR activities for NIC and LID were significantly less ($P < 0.01$) than for PMNs exposed to HBSS only (*t*-test with Bonferroni's correction for multiple comparisons [$n = 5$]).

Table 2. Effect of exposure to various calcium antagonists on neutrophil integrity

Exposure	Concentration (μM)	PMN integrity: Absolute value	PMN integrity: % HBSS control
HBSS		9.53 ± 0.21	100 ± 2.2
LID	10	8.89 ± 0.15	93 ± 1.8
NIC	25	9.44 ± 0.17	99 ± 1.8
NIM	16	8.79 ± 0.56	92 ± 5.9
VER	100	9.72 ± 0.04	102 ± 0.4
FLU	12.5	9.61 ± 0.13	101 ± 1.4

Aliquots of 10 10^6 PMNs were incubated in HBSS ± a calcium antagonist at a concentration of 1.5 IC_{50} (12.5 μM for FLU) for 10 min at 37°, washed twice by centrifugation, resuspended in HBSS, and recounted. Results (means ± SD) are expressed in absolute millions of cells recovered and as a percentage of HBSS (control) cells that were incubated without drugs. Differences between cells exposed to drugs versus HBSS were not significant (*t*-test with Bonferroni's correction for multiple comparisons [$N = 5$]).

trifugation, and then counting the intact cells suspended from the pellet and assaying for LDH activity released into the supernatant fraction. Table 2 indicates that none of the drugs examined resulted in significant loss of PMN number relative to concurrent control cells incubated without drug. Table 3 shows that none of the drugs examined caused significant release of LDH from PMNs, again relative to concurrent control cells incubated without drug compared to a positive control of supernatant fraction obtained from sonicated cells. Essentially identical results were obtained in these cytotoxicity studies in the presence or absence of FMLP (active or quiescent PMNs, NADPH OR). Thus, under the *in vitro* conditions present in the assay system, drug-mediated cytotoxicity does not appear to mimic respiratory burst inhibition.

One additional artifact that would manifest as inhibition of PMN O_2^- production might be free-radical scavenging activity of these drugs. To test this hypothesis, drugs at a concentration of 1.5 IC_{50} (12.5 μM for FLU) were assessed for their abilities to quench O_2^- reduction of cytochrome *c* in a cell-free system containing xanthine and xanthine oxidase. As noted in Table 4, although LID, VER, NIC, and FLU all produced minimal inhibition of cytochrome *c* reduction relative to HBSS alone, none inhibited to the same extent demonstrated with PMNs. That is, at a concentration of 1.5 IC_{50}, a drug should inhibit >50% of the reaction. In this cell-free system only about 14–16% inhibition of cytochrome *c* reduction was ever seen. Thus, it is unlikely that free-radical scavenging activity accounts for the predominant effect of these agents on intact PMN O_2^- synthesis.

To examine specifically the role of extracellular Ca^{2+} on the activity of PMN NADPH OR, reactions were examined in three distinct Ca^{2+} environments, as summarized in Table 5. Control reactions were conducted in complete HBSS (1.26 mM Ca^{2+}, 0.90 mM Mg^{2+}). Initial velocity of FMLP-induced O_2^- synthesis in this complete system was normalized to 100%. When conducted in incomplete HBSS (but without efforts to specifically deplete intracellular neutrophil Ca^{2+}), O_2^- production was about one-third that seen in complete HBSS. The extent of reaction was correspondingly affected. When 5 mM EGTA was added to incomplete HBSS and the reaction was examined, no additional significant inhibition of V_i was noted.

DISCUSSION

Tissue reanimation after an ischemia–reperfusion insult represents the current frontier in resuscitation medicine. Animal models examining cerebral resuscitation and myocardial infarction initiated this field of research which has expanded to include virtually all organ systems. Current evidence supports the notion that the reperfusion injury may be as destructive as the initial anoxic-ischemic event [1–5]. Both ischemia and reperfusion may result in increased cellular accumulation of calcium, which probably pays a pivotal role in cellular morbidity and mortality [14]. A variety of pathologic calcium cascades undoubtedly contribute to the ischemia–reperfusion insult [15]. Accordingly, one class of pharmacologic agents under intense scrutiny with regard to ischemia–reperfusion therapeutics is the

Table 3. Lactate dehydrogenase (LDH) release from neutrophils exposed to various calcium antagonists

Exposure	Concentration (μM)	LDH activity	
		Absolute value	% Sonicated control
Sonicated		15.5 ± 0.70	100 ± 4.5
$HBSS^{++}$		1.72 ± 0.19	11.1 ± 1.2
VER	100	2.23 ± 0.097	14.4 ± 0.6
LID	10	2.13 ± 0.073	13.7 ± 0.5
NIC	25	1.86 ± 0.24	12.0 ± 1.6
FLU	12.5	1.04 ± 0.19	6.7 ± 1.2
NIM	16	2.14 ± 0.11	13.8 ± 0.7

Aliquots of 10×10^6 PMNs were incubated in HBSS ± a calcium antagonist at a concentration of 1.5 IC_{50} (12.5 μM for FLU) for 10 min at 37°, or sonicated (positive control). Cells or membranes were separated from supernatant fractions by centrifugation. Supernatant fractions were assayed for LDH activity. Results are expressed in absolute terms (nmol of lactate oxidized/min/0.10 ml of supernatant) and as a percentage of LDH activity found in the positive control sonicated cell supernatant fraction. Each value reflects the mean ± SD of three assays. No LDH value for drug supernatant fractions was statistically different from the $HBSS^{++}$ supernatant fraction (t-test with Bonferonni's correction for multiple comparisons [N = 5]; for FLU $0.01 < P < 0.02$).

Table 4. Free-radical scavenging activity of various calcium antagonists

Exposure	Concentration of Ca^{2+} channel blocker (μM)	Activity	
		Absolute value	% HBSS control
HBSS		1.03 ± 0.08	100 ± 7.9
NIM	16	1.03 ± 0.06	100 ± 6.0
LID	10	0.87 ± 0.0	84.2 ± 0.0
VER	100	0.87 ± 0.05	85.8 ± 5.3
NIC	25	0.88 ± 0.14	85.8 ± 13
FLU	12.5	0.88 ± 0.03	85.8 ± 3.0

Initial velocity (V_i) $O_2^{\cdot-}$ production, as assessed by cytochrome c reduction, was monitored in a cell-free system containing xanthine and 0.01 units of xanthine oxidase. Calcium antagonists were added to this system at a concentration of 1.5 IC_{50} (12.5 μM for FLU). Inhibition of cytochrome c reduction was monitored for each drug and compared to HBSS as a 100% control. Each value is the mean ± SD of three assays. Activities are presented both in absolute (μmol cytochrome c reduced per min) and relative (% HBSS control) terms. Although each of the drugs tested appeared to exhibit some free-radical scavenging activity, none acted significantly different from control reactions without drugs (t-test with Bonferroni's correction for multiple comparisons [N = 5]).

calcium (channel) antagonists. Although originally thought to simply antagonize the "no reflow" phenomenon, these drugs demonstrate a variety of effects in addition to relaxation of vascular smooth muscle which may include inhibition of protease and phospholipase activation, cytosolic and mitochondrial calcium overloading, prostaglandin H production, xanthine dehydrogenase to xanthine oxidase conversion, erythrocyte rheology alterations, platelet and leukocyte aggregation and phagocyte activation and degranulation [16]. Several members of this diverse drug class (e.g. nicardipine [17], nimodipine [18], lidoflazine [19], flunarizine [20]) have been utilized to ameliorate ischemia–reperfusion injury, even when given after reperfusion.

Oxygen free radicals appear to represent critical mediators of the reperfusion injury, since various free radical scavenging agents such as superoxide dismutase, catalase, dimethyl sulfoxide, α-tocopherol, 5-aminosalicylate and mannitol have been demonstrated frequently to modulate this injury in animal models [e.g. Ref. 21]. Sequestration of free iron by deferoxamine appears to limit non-enzymatic catalyzed oxyradical synthesis and exert a similar protective effect [22]. Experiments analyzing electron spin resonance spectroscopy [23], chemiluminescence [24], accumulation of lipid per-

Table 5. Effect of extracellular calcium on neutrophil NADPH OR initial velocity

Expt.	NADPH OR V_i (% HBSS^{++})		
	HBSS^{++}	HBSS$^{=}$	HBSS$^{=}$ + 5 mM EGTA
A	100 ± 19.1	35.1 ± 6.4*	31.8 ± 6.4*
B	100 ± 7.0	25.6 ± 2.3*	23.2 ± 9.3*
C	100 ± 9.5	40.7 ± 5.4*	37.9 ± 13.5*
Average ± SD	100 ± 11.9	33.8 ± 4.7*	31.0 ± 8.7*

Aliquots of 4×10^5 PMNs were assayed for their ability to generate (FMLP-stimulated) $O_2^{\cdot}$, in complete HBSS (HBSS^{++}), in incomplete HBSS (HBSS$^{=}$), and in HBSS$^{=}$ supplemented with 5 mM EGTA. For each of three experiments, data were normalized to control reactions conducted in HBSS^{++}. Absolute control NADPH oxidoreductase (NADPH OR) initial velocities activities (V_i) were 3.4 ± 0.6, 4.5 ± 0.3, and 4.0 ± 0.4 nmol $O_2^{\cdot}$/min/10^6 PMNs for experiments A, B, and C respectively. Each value is the mean ± SD of three to four determinations of V_i. V_i for reactions conducted in HBSS$^{=}$ and HBSS$^{=}$ + 5 mM EGTA were not different from each other, but were significantly less ($P < 0.001$) than V_i for reactions in HBSS^{++} (*t*-test with Bonferroni's correction for multiple comparisons [N = 2]).

oxidation products [25], *in situ* nitroblue tetrazolium reduction [26], and loss of reduced glutathione [27] provide direct evidence for production of oxyradicals in ischemia/reperfused tissue.

Synthesis of these toxic species is multifactorial. Xanthine oxidase is undoubtedly a major contributor [1] but catecholamine reoxidation, synthesis of prostaglandin H, and inflammatory cell infiltration represent additional important sources of oxyradicals in this setting [28]. With initial endothelial membrane damage (e.g. lipid peroxidation), a variety of neutrophil chemotactic factors including platelet activating factor, leukotriene B_4, activated complement, and thromboxane A_2 may be generated. Early accumulation of PMNs in post-ischemic tissue has been demonstrated repeatedly and may relate to the degree of ischemia [3, 5]. Whereas downstream concentration of a chemoattractant may initiate directed PMN migration, concentration at the source may be of sufficient strength to stimulate the phagocyte respiratory burst [6], an important source of oxygen free radicals [7], which may amplify the original injury [7]. Activation of NADPH OR represents the premier event in the PMN respiratory burst, since this membrane bound flavoprotein/cytochrome complex is responsible for synthesis of the parent oxyradical species, namely $O_2^{\cdot}$. Production of other oxyradical species (H_2O_2, $HO^{\cdot}$, HO^-, $HClO^-$, singlet oxygen, chloroamines) results from participation of enzymatic (myeloperoxidase, superoxide dismutase) and non-enzymatic (Haber–Weiss, Fenton reactions) processes [28]. Maximum activity of NADPH OR in both intact cells as well as purified PMN membrane requires the presence of Ca^{2+} [8, 9]. We hypothesized that one effect of Ca^{2+} antagonists may be suppression of PMN $O_2^{\cdot}$ release, by inhibition of PMN NADPH OR.

Results of the present study indicate that selective calcium channel antagonists inhibit PMN NADPH OR (and hence $O_2^{\cdot}$ production) with individual specificities reflected in IC_{50} values ranging between 7 and 45 μM for LID, NIM, NIC and VER. No inhibition was noted for FLU at concentrations ≤ 25 μM.

Plasma levels of near 3 μM have been observed in rats after an oral 3 mg/kg dose of nicardipine [29]. Plasma levels near 0.4 μM have been seen in humans given a smaller intravenous bolus [30]. This drug is extracted into the brain, especially ischemic brain [31]. By increasing the dose or prolonging the exposure, a pharmacologic effect of NIC on human PMNs *in vivo* may be possible (IC_{50} NIC = 12 μM).

When utilized in a study examining neurologic outcome after subarachnoid hemorrhage, patients receiving 2.1 mg/kg/day of oral nimodipine exhibited plasma levels near 0.02 μM without any adverse effects [32]. Oral LD_{50} doses in various animal species are at least 1000 times this dose [33]. Acute peak plasma levels of NIM in humans following a 50 μg/kg bolus approach 0.25 μM [34]. It may be feasible to safely increase plasma NIM levels towards those required to inhibit some PMN $O_2^{\cdot}$ synthesis (IC_{50} NIM = 20 μM). An advantage of the dihydropyridine group of calcium antagonists (NIC, NIM) is their relative lack of adverse inotropic, chronotropic and dromotropic effects [35].

Canine dose–response data for lidoflazine indicate that a 10 mg/kg dose (N = 9) results in a peak plasma level of 3.1 ± 0.12 μM [36]. Rats given 20 mg/kg of the related *p*-fluorobiphenylmethane derivative flunarizine, exhibit brain concentrations near 6 μM [37]. Steady-state plasma FLU levels in rats given 10 mg p.o. per day were only 0.1 to 0.2 μM, but much higher levels were assayed in brain tissue. Like hydropyridines, *p*-fluorobiphenylmethane derivatives exhibit less adverse hemodynamic effects (hypotension, heart block) as compared to benzeneacetonitriles (VER). Accordingly, *in vivo* concentrations near IC_{50} LID = 7 μM may be approachable.

Alternatively, clinically relevant plasma concentrations of VER are in the 0.05 to 0.5 μM range. Plasma concentrations up to 1.8 μM have been noted in asymptomatic individuals, but heart block and hypotension become common with higher concentrations [38]. Raising the VER concentration to

that effective in inhibiting PMN $O_2^{\cdot-}$ production (IC_{50} VER = 45 μM) would likely result in profound adverse hemodynamic effects.

VER has been examined previously for several effects on PMNs. Other investigators have ascertained that VER increases lag time preceding $O_2^{\cdot-}$ synthesis [39]. In the present study at concentrations 1–2 IC_{50}, LID, NIC, and VER each significantly prolonged lag time but only NIC demonstrated a consistent concentration–response effect (see Table 1). Dose-dependent inhibition of $^{45}Ca^{2+}$ uptake, transmembrane potential changes, chemotaxis, respiratory burst activation and O_2 consumption by Ca^{2+} antagonists have been reported [40–46]. When incubated with VER or nifedipine, PMN bacterial killing is impaired although phagocytosis is preserved [42]. Calcium antagonists do not appear to alter plasmalemma binding of respiratory burst stimuli [44, 46]. Inhibition of lymphocyte proliferation [47], macrophage activation [48], and platelet function [49] has similarly been described for VER, nifedipine and diltiazem. The mechanism of inhibition of cell function by these agents may be multifaceted [41, 44, 45, 50]. As concluded from Table 5, calcium antagonists were noted to inhibit $O_2^{\cdot-}$ synthesis beyond that of calcium depletion in the reaction mixture (no efforts made to specifically deplete intracellular Ca^{2+}). Therefore, it is likely that their mode of action involves more than simply blocking the flux of calcium across the PMN plasmalemma. More important effects of Ca^{2+} antagonists relative to the neutrophil may involve actions at intracellular sites such as cyclic AMP phosphodiesterase [41, 51], Ca^{2+}-activated phospholipid-dependent protein kinase C [e.g. Refs 41 and 45], calmodulin [52], or intracellular membrane Ca^{2+} binding [41, 43, 45].

Concentration–response inhibition of FMLP-stimulated respiratory burst activity by VER noted in the present study compares to that seen by other investigators [40, 41]. When phorbol myristate acetate is used as the PMN stimulus, significantly higher concentrations of VER (IC_{50} 300 μM) are required to inhibit $O_2^{\cdot-}$ synthesis [45]. Note that PMA activation involves stimulation of a protein kinase and is independent of plasmalemma calcium fluxes, but still subject to Ca^{2+} antagonist inhibition.

LID, FLU, NIC, and NIM were investigated because they have been utilized most frequently in ischemia–reperfusion research. Both *p*-fluorobiphenylmethane derivatives (LID, FLU) and hydropyridines (NIC, NIM) [35] have less pronounced acute hemodynamic effects than the benzeneacetonitriles (VER), although long-term use of LID has been associated with ventricular arrhythmias. Interestingly, LID, NIC and NIM all displayed lower IC_{50} values (μM) than VER, relative to PMN $O_2^{\cdot-}$ inhibition.

At the concentrations examined, these drugs do not appear to be cytotoxic (Tables 2 and 3) [44]. Moreover, inhibition appears to be at least partially reversible (Fig. 3) by simple drug washout. Such considerations would be important in designing *in vivo* models to examine the role of Ca^{2+} antagonists and PMNs in ischemia–reperfusion injury. Selection of an appropriate therapeutic window to curtail PMN autoinjury while avoiding nosocomial infection secondary to acquired PMN immunodeficiency would be the goal. Reversibility of inhibition is required to accurately titrate this effect.

Each of the drugs, except NIM, displayed nominal activity as a free-radical scavenging agent utilizing the xanthine/xanthine oxidase $O_2^{\cdot-}$-generating system. Such free-radical scavening activity has been demonstrated previously for FLU, VER, diltiazem and nifedipine utilizing other cell-free radical-generating systems [37]. However, free-radical scavenging is more efficient at drug concentrations significantly above IC_{50} values determined in the present study.

Animal models have not confirmed a consistent source of oxyradical production which may be implicated in ischemia/reperfusion pathogenesis. For example, some investigators have variously failed to demonstrate a beneficial effect of allopurinol administration or neutrophil depletion on histopathology or outcome. Such findings may reflect the relative contributions of primary and secondary reperfusion injuries. Although xanthine oxidase may catalyze an initial $O_2^{\cdot-}$-mediated reperfusion injury, PMNs characteristically orchestrate a secondary amplification injury. With regards to PMN-mediated reperfusion autoinjury, several modalities are operative: (1) leukoagglutination may result in capillary plugging directly; (2) release of PMN elastase, collagenase, and phospholipase may result in hydrolytic injury (oxyradicals cause oxidative fragmentation of α_1-antitrypsin, the normal host protease defense); and (3) toxic oxygen radicals may mediate lipid, protein and nucleic acid oxidation. Each of these aspects of PMN inflammation amplification is dependent on calcium ion fluxes.

The present investigation concludes that Ca^{2+} antagonists in the micromolar concentration range can inhibit *in vitro* production of $O_2^{\cdot-}$ by PMNs by inhibiting the activation and activity of PMN NADPH OR. Indirect actions such as activation/desensitization, free-radical scavenging, and cytotoxicity do not appear to be important drug effects. Attaining plasma levels of some of these drugs in the range of IC_{50} without adverse hemodynamic effects may be feasible. Since PMNs accumulate rapidly in post-ischemic tissue, further *in vivo* investigation of these agents examining their utility to prevent PMN-associated host autoinjury in the setting of ischemia–reperfusion seems appropriate.

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