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Effects of perhexiline and nitroglycerin on vascular, neutrophil and platelet function in patients with stable angina pectoris☆

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Abstract

Perhexiline, a "metabolic" anti-anginal agent currently under investigation in management of congestive heart failure and acute coronary syndromes improves platelet nitric oxide responsiveness in patients with impaired responsiveness. The current study investigated possible interactions between perhexiline and the nitric oxide donor nitroglycerin on arterial stiffness, neutrophil superoxide release and on platelet nitric oxide responsiveness. Patients ($n=39$) with stable angina pectoris, awaiting cardiac catheterization were randomized to additional perhexiline or unchanged drug therapy; all patients received nitroglycerin infusion for 2 h. Vasomotor responses to perhexiline and combined perhexiline/nitroglycerin were examined using changes in augmentation index, measured via applanation tonometry. Neutrophil superoxide release was measured ex vivo utilizing lucigenin mediated chemiluminescence and effect of perhexiline on inhibition of platelet aggregation by sodium nitroprusside was also measured. Perhexiline alone did not affect augmentation index, neutrophil superoxide release, or ex vivo platelet sodium nitroprusside response. Nitroglycerin decreased augmentation index ($P<0.01$) and superoxide release ($P<0.05$). Magnitude of inhibition of superoxide release was significantly enhanced by perhexiline pre-treatment ($P<0.05$); however perhexiline had no effect on magnitude of vasomotor response to nitroglycerin. In conclusion, perhexiline exerts no effects on arterial stiffness and does not potentiate nitroglycerin induced dilatation. In patients with normal platelet function perhexiline does not affect platelet nitric oxide responsiveness. In vivo low dose nitroglycerin inhibits neutrophil superoxide release; this effect is potentiated by pre-treatment with perhexiline. These "anti-inflammatory" effects of nitroglycerin may contribute to utility in acute coronary syndromes and congestive heart failure.

Keywords: Nitroglycerin; Perhexiline; Superoxide release

1. Introduction

A number of recent investigations have addressed the possibility that maintenance of biological effect of nitric oxide is of major prognostic importance in individuals with a variety of cardiovascular disease states. Three different mechanisms contribute to the loss of biological effect of nitric oxide in various cardiovascular disease states. These include: clearance of nitric oxide via scavenging of nitric oxide by superoxide (Chirkov et al., 1999; Laursen et al., 1997; Rajagopalan et al., 1996), loss of effect of nitric oxide due to interruption of downstream signalling pathways (Bauersachs et al., 1998; Watanabe et al., 1998) and inhibition of the production of nitric oxide by inhibition of nitric oxide synthase (Ito et al., 1999; Jin and D'Alecy, 1996). The endogenous inhibitor of nitric oxide synthase, asymmetric dimethyl arginine, is a potent independent prognostic marker (Schnabel et al., 2005); elevation of asymmetric dimethyl arginine levels in blood tend to reflect redox-sensitive inactivation of the enzyme responsible for clearance (Sydow and Munzel, 2003). Furthermore, the known adverse prognostic implications of "endothelial dysfunction" have recently been extended by demonstration that patients with hypertension, angina and congestive heart failure often

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exhibit decreases in responsiveness to nitric oxide (as well as decreased generation of nitric oxide) in both blood vessels and in platelets (Celermajer et al., 1992; Chirkov et al., 1999; Schachinger et al., 2000). This phenomenon of nitric oxide resistance is also an adverse prognostic marker (Schachinger et al., 2000; Willoughby et al., 2005). Therefore, it is possible that agents that augment tissue responsiveness to nitric oxide will thereby exert beneficial effects on cardiovascular outcomes, as recently demonstrated with the angiotensin converting enzyme inhibitor perindopril (Chirkov et al., 2004), and possibly statins (John et al., 2001).

Perhexiline is a "metabolic" agent that is utilized primarily in the management of otherwise refractory angina pectoris (Cole et al., 1990). Previous studies (reviewed in Lee et al., 2004) have suggested that its major mechanism of therapeutic effect is inhibition of long-chain fatty acid oxidation, with a secondary stimulation of glucose utilization and an increase in myocardial efficiency (Jeffrey et al., 1995). However, a number of interesting additional findings suggest that the effects of perhexiline may be more complex, and that the clinical spectrum of utility may be more extensive. For example, in patients with refractory symptoms after presentation with acute coronary syndromes, perhexiline in therapeutic doses appears to accelerate resolution of ischemic episodes (Stewart et al., 1996), and also sensitizes platelets to nitric oxide (Willoughby et al., 2002). Furthermore, recent animal/tissue studies from our laboratory have suggested that perhexiline inhibits superoxide release; especially from tissues other than intact blood vessels (Kennedy et al., 2006) and that an increment in myocardial efficiency is induced by perhexiline in the absence of ischemia, and cannot be solely explained by inhibition of fatty-acid oxidation (Kennedy et al., 2000).

Perhexiline has been considered essentially hemodynamically inert (apart from very weak calcium antagonist properties (Barry et al., 1985; Fleckenstein-Grun et al., 1978)), and does not appear to increase incidence of headaches in organic nitrate-treated patients. Nevertheless, a study in patients with congestive heart failure has shown that perhexiline considerably improves hemodynamic status (incrementally over conventional therapy) and that this improvement is independent of the ischemic/non-ischemic basis for congestive heart failure (Lee et al., 2005).

To date, the putative effects of perhexiline on responses to nitric oxide and superoxide release have not been examined in humans with the exception of the previous study in platelets (Willoughby et al., 2002). We therefore performed a clinically based study to test, in a population of patients with stable angina pectoris, the following hypotheses:

1. That perhexiline exerts neither direct nor nitric oxide potentiating effects on vasomotor tone.
2. That perhexiline inhibits neutrophil superoxide release in vivo, and that this effect is potentiated by therapeutically infused nitric oxide donors.
3. That the effects of perhexiline include potentiation of platelet responsiveness to nitric oxide donors in a population with mild ischemic symptoms.

2. Materials and methods

2.1. Patient selection

Patients were considered for entry on the basis of clinical diagnosis of either chest pain of uncertain aetiology or of stable angina pectoris (Canadian Class II or III) not receiving long-term prophylactic nitrate therapy. In all cases elective cardiac catheterization was planned. Exclusion criteria were: treatment with long acting nitrate therapy or previous adverse reactions to organic nitrates, perhexiline or other "metabolic" anti-anginal agents, utilization of thienopyridine anti-aggregatory agents, clinically significant hepatocellular disease or findings of hemodynamically significant left main coronary stenosis. The protocol was approved by the institutional Ethics of Human Research Committee and informed consent was obtained prior to study entry.

2.2. Experimental protocol

The protocol is outlined in Fig. 1. Patients ($n=39$) were randomized to receive perhexiline (400 mg/day) or no additional therapy 3 days prior to catheterization, with blinding of all study personnel to treatment regimen; this 3 day pre-treatment facilitates attainment of therapeutic perhexiline levels (Horowitz et al., 1986). Intravenous infusion of nitroglycerin (5 μg/min) utilizing non-adsorptive delivery tubing and glass reservoir was commenced immediately prior to angiography and continued for at least 2 h. Pulse wave analysis was performed and neutrophil superoxide release measured prior to randomization, immediately prior to catheterization and after two hours of nitroglycerin infusion. Platelet studies were performed prior to randomization and immediately prior to catheterization. To investigate the possibility of an effect of the angiogram itself, a comparison group ($n=6$) underwent angiography with no additional treatment: neutrophils superoxide release was measured before and two hours after angiography.

2.3. Applanation tonometry

Apparent arterial stiffness was measured serially (as previously described (Kelly et al., 2005)) utilizing applanation tonometry, in which radial artery pressure wave forms are recorded using a high fidelity micromanometer (SPT-301B;

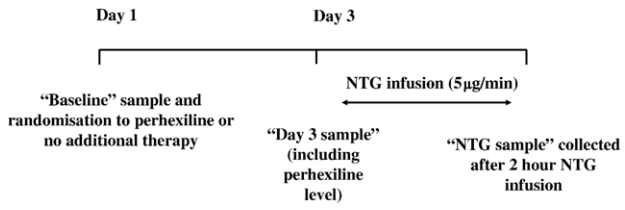

Fig. 1. Outline of study protocol. Baseline samples were taken three days prior to cardiac catheterization and patients were then randomized in a single blind design to perhexiline or no perhexiline. Day three, samples were taken prior to catheterization. Nitroglycerin (NTG) infusion was commenced at the time of catheterization and NTG sample was taken after two hours of infusion.

Millar instruments, Houston, TX, USA) from the radial artery of the dominant arm. Pulse wave analysis (Sphygmocor version 7, Atcor Medical, Sydney, NSW, Australia) was then used to generate corresponding central waveforms via a validated transfer function. Augmentation index, a measure of systemic arterial stiffness (Vlachopoulos et al., 2001), was determined from the central waveform by expressing the difference between the first and second systolic peaks as a percentage on the total pulse pressure; measures of augmentation index were corrected for variations in heart rate.

2.4. Neutrophil superoxide production

This was assessed via a modification of our previously published method (Willoughby et al., 2002). Briefly, venous blood was anti-coagulated with EDTA. Plasma was separated by centrifugation and retained. Red blood cell layer and buffy coat was diluted with Hanks' balanced salt solution and neutrophils were separated by centrifugation across Lymphoprep density gradient (Axis-Shield, Oslo, Norway). Hypotonic lysis (155 mM NH_4Cl, 100 μM Na_2EDTA, 10 mM $NaHCO_3$) was used to remove red cells and isolated neutrophils were washed in Hanks' balanced salt solution and re-suspended in platelet free plasma at 1.7×10^6 cells/mL. Neutrophils were stimulated with the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, 1 μM). Superoxide release was measured at 37 °C by lucigenin (bis-*N*-methylacridinium nitrate, 10 μM) mediated chemiluminescence at 20 s intervals for 5 min using a luminometer (6100 Pico-lite luminometer, Packard, Illinois). Data are expressed as area under the curve (0–5 min).

2.5. Platelet aggregation studies

Platelet studies were conducted as previously described (Chirkov et al., 1999). Briefly, citrated venous blood was diluted 2 fold with 0.9% saline. Aggregation in response to 1 μM ADP was measured using a dual channel impedance aggregometer (Model 560, ChronoLog) and recorded as maximal aggregation (in ohms). Inhibition of aggregation by sodium nitroprusside was determined by measuring maximal aggregation to ADP after 1 min pre-incubation with sodium nitroprusside and results were expressed as percent inhibition of maximal aggregation by sodium nitroprusside.

2.6. Chemicals

Nitroglycerin was obtained from Mayne Pharma (Melbourne, Australia). Hanks' balanced salt solution was purchased from Life Technologies (Maryland, USA). Perhexiline, *N*-formyl-methionyl-leucyl-phenyl alanine, bis-*N*-methylacridinium nitrate (lucigenin), sodium nitroprusside and ADP were obtained from Sigma Chemical Company (St. Louis, MO, USA).

2.7. Data analysis

Normally distributed data were analyzed by two-way analysis of variance (ANOVA). Where raw data were not normally distributed data, changes over time (which were normally distributed) were compared between the two groups by *t*-tests, and changes within groups were analyzed using Wilcoxon matched pairs test. Patient characteristics were compared using Fisher's exact test for categorical data or Student *t*-tests for continuous data.

3. Results

3.1. Patient characteristics

As seen in Table 1, patients in both groups were well matched for risk factor profile and drug treatment. The majority of patients were receiving one prophylactic anti-ischemic agent (either β-adrenoceptor antagonist or non-dihydropyidine calcium antagonists). Most patients were also treated with statins and 49% were receiving either angiotensin converting enzyme inhibitors or angiotensin receptor antagonists. The majority of patients had limited coronary artery disease, with patients randomized to perhexiline having a higher ($P<0.05$) incidence of 3 vessel disease. All patients had well preserved left ventricular systolic function and normal pulmonary capillary wedge pressures (data not shown).

Plasma perhexiline concentrations after 3 days of treatment were 0.32±0.01 μg/mL (therapeutic range 0.15–0.6 μg/mL (Horowitz et al., 1986)). All patients tolerated both perhexiline and nitroglycerin without symptomatic side-effects over the study period.

3.2. Vascular studies

The effects of nitroglycerin infusion and its interaction with perhexiline therapy are summarized in Fig 2. As previously described (Kelly et al., 2005) nitroglycerin, even at this low

Table 1
Characteristics and drug treatment of patients in control and perhexiline groups

	Control group $n=22$	Perhexiline group $n=17$
Risk factors		
Age	57±0.6	62±0.6
Male gender	13 (59%)	10 (59%)
Current smoking	5 (23%)	5 (29%)
Hypertension	12 (55%)	10 (59%)
Hypercholesterolemia	18 (82%)	13 (76%)
Diabetes mellitus	4 (18%)	3 (18%)
Drug treatment		
Statin	16 (73%)	11 (65%)
β-Adrenoceptor antagonist	8 (36%)	3 (18%)
Aspirin	21 (95%)	17 (100%)
ACE inhibitor/AT-1 receptor antagonist	10 (46%)	9 (53%)
Ca^+ antagonist	14 (64%)	13 (76%)
Number of stenosed/occluded coronary arteries[†]		
0	3 (14%)	0
1	9 (41%)	6 (35%)
2	10 (45%)	5 (29%)
3	0	6 (35%)*

[†]Significant stenosis=<50% stenosis in a major epicardial vessel, *$P<0.05$.

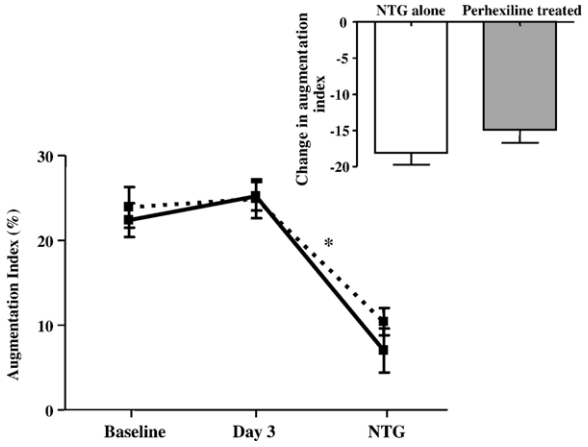

Fig. 2. Effect of perhexiline alone and in combination with nitroglycerin (NTG) on augmentation index, shown as mean±S.E.M. Solid line represents nitroglycerin alone and the broken line represents perhexiline treated. Perhexiline alone had no significant effect on augmentation index. *Nitroglycerin infusion significantly reduced augmentation index in both groups of patients ($P<0.01$, $F=251.8$, ANOVA). There was no significant difference in the magnitude of this change between groups (inset).

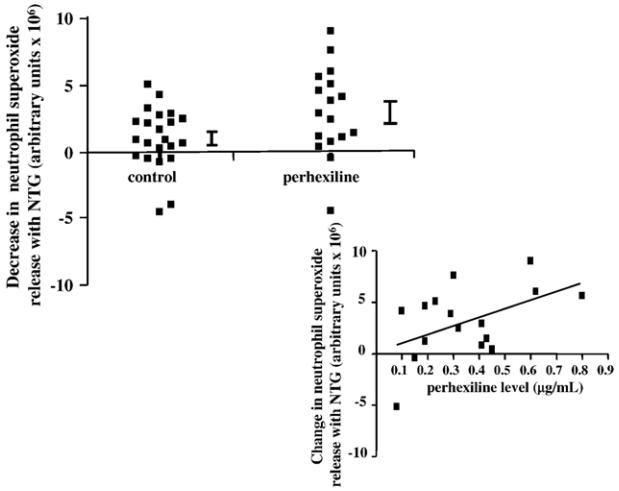

Fig. 3. Decrease in neutrophil superoxide release during nitroglycerin (NTG) infusion in the presence and absence of perhexiline pre-treatment (error bars represent inter-quartile range). Nitroglycerin alone significantly decreased neutrophil superoxide release ($P<0.05$, Wilcoxon matched pairs test) and the magnitude of this effect was significantly greater in the presence of perhexiline pre-treatment ($P<0.05$, unpaired t-test). Inset: There was a non-significant trend ($r^2=0.24$, $P=0.053$) toward direct relationship between neutrophil response to nitroglycerin and plasma perhexiline concentrations.

infusion rate, induced a significant fall in augmentation index ($P<0.01$, $F=251.8$). This was paralleled by a mean decrease in systolic blood pressure of 24±0.6 mm Hg. However there was no significant effect of perhexiline alone on augmentation index or on systolic blood pressure, nor was there a significant interaction between augmentation index response to nitroglycerin and perhexiline therapy (see Fig. 2 inset), consistent with the hypothesized "non-vasoactive" pharmacology of perhexiline. Furthermore, in patients with less than median responses to nitroglycerin (relatively "nitric oxide resistant" at the vascular level) there was also no evidence of potentiation of nitroglycerin effect by perhexiline.

3.3. Neutrophil superoxide release

Changes in fMLP-stimulated neutrophil superoxide release during nitroglycerin infusion are summarized in Fig. 3. Raw data are expressed as area under the superoxide release: time curve from 0 to 5 min in arbitrary units $\times 10^6$ and median data (and inter-quartile range) are reported as data were not normally distributed. Perhexiline treatment for 3 days had no significant effect on superoxide release (control: 5.080 (2.84–10.03) to 3.755 (2.69–6.78), perhexiline: 3.635 (2.54–7.28) to 4.510 (1.97–9.99)). However nitroglycerin induced a marked inhibition of superoxide release (see Fig. 3, $P<0.05$ in both groups, Wilcoxon matched pairs tests). The mean decrease in neutrophil superoxide release induced by nitroglycerin was approximately 3 times greater in the perhexiline group than in the control group. Furthermore (inset Fig. 3) there was a correlation of borderline significance ($r^2=0.24$, $P=0.053$) between the magnitude of nitroglycerin effect on neutrophil superoxide release and plasma perhexiline level. Administration of contrast agents and heparin without nitroglycerin during angiography ($n=6$) did not affect neutrophil superoxide release (mean delta of 0.92±0.55, $P=$NS).

3.4. Platelet studies

Platelet responsiveness to nitric oxide was measured ex vivo as previously described (Chirkov et al., 1999) by measuring inhibition of ADP-induced aggregation by sodium nitroprusside, before and after three days of perhexiline treatment. Platelet responsiveness to sodium nitroprusside was unchanged during the three day study period in either the perhexiline treated or control group (2-way ANOVA, control: 47.0±5.8% to 52.1±7.1%, perhexiline: 57.8±5.8% to 62.8±5.8%, see Fig 4). This result was not affected by changes in extent of aggregation as there was no change in the extent of aggregation in either group during the study period (control: 9±0.7 Ω to 8±0.7 Ω, perhexiline: 8.7±0.8 Ω to 8.0±1.0 Ω). Patients randomized to perhexiline exhibited slightly better platelet responses to sodium nitroprusside than the control group, although this difference was not a significant difference from controls. However, of interest is that in this patient group platelet nitric oxide responsiveness was relatively well preserved as

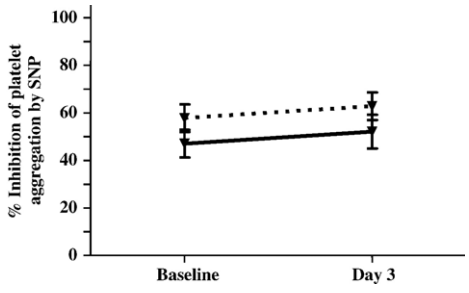

Fig. 4. Effect of perhexiline on platelet nitric oxide responsiveness shown as mean±S.E.M. Solid line represents nitroglycerin alone and the broken line represents perhexiline pre-treated. There was no change in response to the nitric oxide donor sodium nitroprusside in either group over the 3 day study period.

mean inhibition of aggregation by sodium nitroprusside in normal volunteers is 66±19% (Chirkov et al., 1999).

4. Discussion

These investigations were undertaken primarily to establish that perhexiline, while having no vasomotor effect, potentiates non-vasomotor effects of nitroglycerin. Indeed the results revealed no evidence that perhexiline exerts clinically significant vasomotor effects either alone or in combination with nitroglycerin. However, perhexiline markedly potentiated the effects of a low infusion rate of nitroglycerin in suppressing neutrophil superoxide release despite having no effect in monotherapy. Perhexiline exerted no effects on platelet responsiveness to nitric oxide in this patient group. A further critically important finding was related to the effect of nitroglycerin on neutrophil superoxide release: 5 μg/min nitroglycerin, representing a very low clinical infusion rate (Arstall et al., 1995), suppressed superoxide release by approximately 20%.

The finding that perhexiline lacks intrinsic or nitric oxide-potentiating effects on augmentation index was consistent with previous clinical experience with the drug: neither development of hypotension nor precipitation of headaches in nitrate-treated patients are recognized side effects of the drug. Nevertheless, perhexiline does exert vasodilator effects in vitro in some animal models (Fleckenstein-Grun et al., 1978; Klaus and Guttler, 1978), probably via its weak L-type calcium antagonist effects. However the lack of significant potentiation of sodium nitroprusside-induced inhibition of platelet aggregation by perhexiline was unexpected. Willoughby et al. (2002) have previously shown that perhexiline potentiates sodium nitroprusside and nitroglycerin effects on platelet aggregation in cohorts of patients with both stable and unstable angina. The lack of significant effect in the current study probably reflects the relatively normal baseline platelet function in the current study group, whereas the previous cohort was markedly hyporesponsive to nitric oxide donors prior to initiation of perhexiline therapy.

This is the first in vivo human study to document the effects of nitroglycerin or any other organic nitrate in suppressing superoxide release from neutrophils. This is potentially a most important finding, which may improve understanding of the therapeutic potential of all agents releasing or potentiating nitric oxide, not just organic nitrates. The observed suppression of superoxide release was detected ex vivo, and cannot be attributed to "scavenging" of released superoxide by the nitric oxide$^{\cdot}$ radical, given the very short half life of nitroglycerin. While the biochemical mechanism(s) of the effect was not evaluated, previous investigators have raised the possibility that nitric oxide may exert "anti-inflammatory" effects, and it has been shown that high concentrations of nitric oxide in vivo inhibit NAD(P)H oxidase, a major source of superoxide release (Klaus and Guttler, 1978). The current data are consistent with previous reports that nitroglycerin attenuates oxidative stress induced by nitrilotriacetate in mice (Iqbal et al., 2003) although the effects of low doses of nitroglycerin were not explored in that study. Another study in dogs demonstrated that nicorandil, a nitrate and potassium channel opener, inhibited neutrophil superoxide release (Pieper and Gross, 1992), however in this study it is unclear which mechanism of action of nicorandil is responsible for this inhibition (Mizumura et al., 1995). It remains to be determined whether the currently observed nitric oxide effect is modulated by activation of soluble guanylate cyclase (although there is some evidence that activation of soluble guanylate cyclase inhibits neutrophil activation and superoxide release from neutrophils (Wang et al., 2002)), and whether this acute nitroglycerin effect is potentially subject to tolerance induction. Clinically, the finding is of particular interest as regards the management of acute coronary syndromes, in which inflammatory response plays a pivotal role (Buffon et al., 2002). Disturbance of coronary artery plaques (during angioplasty) has been shown to result in activation of neutrophils in vivo (van der Wal et al., 1994) and neutrophil infiltrate has been found at the site of ruptured plaques (Naruko et al., 2002). Activation of inflammatory cells has also been shown to contribute to impairment of endothelial function (Sugano et al., 2005). Interestingly, low dose isosorbide mononitrate, which has no beneficial effect on 35 day mortality post myocardial infarction in the ISIS-4 study, markedly reduced day 1 mortality (ISIS-4 investigators, 1995) via an unknown mechanism. Furthermore, the combination of hydralazine and isosorbide dinitrate was recently shown, in the African-American Heart Failure Trial (Taylor et al., 2004), to improve prognosis in patients with congestive heart failure, a condition characterized by activation of inflammatory processes (Ellis et al., 2000). Our current findings raise the possibility of an additive or synergistic effect between hydralazine, which inhibits NAD(P)H oxidase in vascular tissue (Munzel et al., 1996) and organic nitrates, which can now be seen to inhibit high-capacity neutrophil superoxide release mechanisms.

As regards potentiation of effects of nitroglycerin on neutrophil superoxide release by perhexiline, only fragmentary mechanistic data are currently available. Perhexiline inhibits superoxide release from neutrophils in vitro (Willoughby et al., 2002), but the interaction with nitroglycerin/nitric oxide has not been investigated in this system. Irrespective of the underlying mechanism(s) of this interaction, these findings are very relevant to the evolving role of this agent in management of acute myocardial ischemia (Philpott et al., 2004) and possibly heart failure (Lee et al., 2005).

5. Limitations

A number of important issues have not been addressed in the current study. Neutrophil studies did not examine mechanisms of responses to nitroglycerin or perhexiline, concentration response relationships or potential attenuation of nitroglycerin effect due to nitrate tolerance. It would also be of interest to compare the interaction between perhexiline and nitroglycerin in a population with a high prevalence of platelet nitric oxide resistance (such as patients with severely symptomatic stable angina or unstable angina (Willoughby et al., 2002)) in order to evaluate the relative extents of the perhexiline–nitroglycerin interactions in platelets and neutrophils. Furthermore, it remains possible that perhexiline might exert vasomotor effects at the

level of the large arteries, venous circulation and/or other vessels not represented by changes in augmentation index.

6. Conclusions

The current study has demonstrated that perhexiline is vasoactively inert both as monotherapy and when combined with intravenous nitroglycerin, and that in this patient cohort with normal platelet function that perhexiline has no effect on platelet nitric oxide responsiveness. However the study also demonstrates for the first time that in vivo administration of low infusion rates of nitroglycerin inhibits neutrophil superoxide release, and that this effect is potentiated by pre-treatment with perhexiline.

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