Mitoquinone (mitoQ) exerts antioxidant effects independent of mitochondrial targeted effects in phorbol-12-myristate-13-acetate (PMA) or N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) stimulated polymorphonuclear leukocyte (PMN) superoxide (SO) release

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Introduction

PMNs have been identified as one of the main mechanisms underlying ischemia reperfusion (I/R) injury. Their tendency to injure the myocardium, coronary endothelium, and myocytes is caused by proinflammatory mediators such as tumor necrosis factor α and interleukin 8. PMNs contain an extensive cytotoxic arsenal and their potential to destroy tissue is caused by degranulation and generation of reactive oxygen species (ROS). The activation of the PMN membrane-associated NADPH oxidase system by proinflammatory cytokines, C5a, platelet activating factor, and particulate stimuli initiates a respiratory burst characterized by a marked increase in cellular oxygen consumption and the generation of SO anions. Protein kinase C (PKC) activation of PMN NADPH oxidase is essential to generate PMN SO release (1). fMLP peptide activates PMN chemotactic receptor to stimulate NADPH oxidase via PKC. PMA is a lipid soluble broad spectrum PKC agonist that directly activates PKC (Fig. 1). Inhibition of PMN SO release attenuates inflammation mediated vascular injury (e.g. I/R).

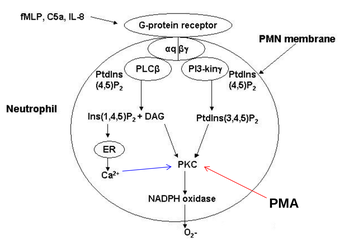

Figure 1. Schematic representation of PKC activation generating SO release in a PMN. PMN chemotactic G-protein receptors are activated by fMLP, complement C5a, and IL-8. The G-protein subunits αq and βγ disassociate after stimulation and activate phospholipase C beta (PLCβ) and phosphatidyl inositol-3-kinase gamma (PI-3kinγ) to produce inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_2$) plus diacylglycerol (DAG) and $PtdIns(3,4,5)P_2$ respectively from phospholipids phosphatidylinositol 4,5 bisphosphate ($PtdIns(4,5)P_2$). $Ins(1,4,5)P_2$ stimulates Ca^{2+} release from the endoplasmic reticulum (ER). Ca^{2+}/DAG and $PtdIns(3,4,5)P_2$ directly activate PKC. PMA directly activates PKC. Activated PKC phosphorylates NADPH oxidase to release SO anion (O_2^-) Adapted from (1).

Ubiquinones, coenzyme Q (CoQ), are stoichiometrically in excess of electron-transfer chains, and it has been suggested that in addition to their role in mitochondrial respiration, ubiquinones in their reduced form, ubiquinols, may act as free radical scavengers. MitoQ, a mitochondria-targeted CoQ antioxidant analog, dose-dependently (1-20 μM) restored post-reperfused cardiac function and reduced infarct size in isolated perfused rat hearts subjected to I/R (2). Accumulation of ubiquinol within mitochondria is necessary for the efficacy of mitoQ in limiting myocardial I/R injury (3). Given that NADPH oxidase is the principal source of PMN SO release, we speculated that mitoQ may exert some of its antioxidant effects independent of the mitochondria.

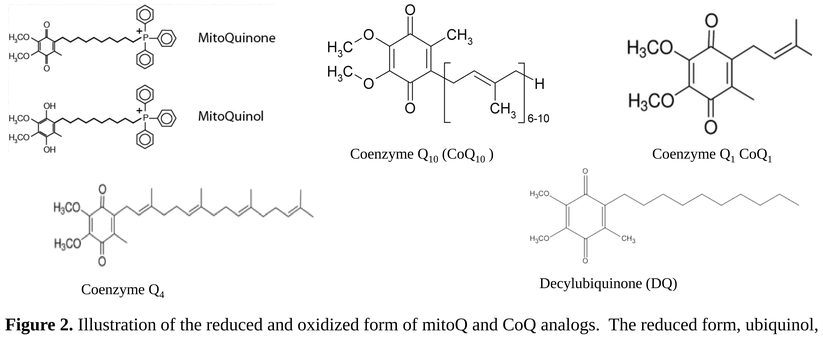

Figure 2. Illustration of the reduced and oxidized form of mitoQ and CoQ analogs. The reduced form, ubiquinol, acts as an antioxidant free radical scavenger via hydrogen atom transfer.

Hypothesis

We hypothesized that MitoQ and other CoQ analogs would dose-dependently attenuate both PMA and fMLP induced SO release in PMNs without affecting cell viability.

Methods

Isolation of PMNs

Male Sprague-Dawley rats (350–400 g, Charles River, Springfield, MA), used as PMN donors, were anesthetized with isoflurane and given a 16 ml intraperitoneal injection of 0.5% glycogen (Sigma Chemical) dissolved in PBS. Rats were reanesthetized with isoflurane 16–18 h later, and the PMNs were harvested by peritoneal lavage in 30 ml of 0.9% NaCl, as previously described (4). The peritoneal lavage fluid was centrifuged at 200 g for 10 min at 4ºC. The PMNs were then washed in 20 ml PBS and centrifuged at 200 g for 10 min at 4ºC. Thereafter, the PMNs were resuspended in 2.5 ml PBS and concentration was calculated. The PMN preparations were >90% pure and >95% viable according to microscopic analysis and exclusion of 0.3% trypan blue, respectively.

Measurement of SO Release From Rat PMNs

The SO release from PMNs was measured spectrophotometrically (model 260 Gilford, Nova Biotech; El Cajon, CA) by the reduction of ferricytochrome c (4). The PMNs (5×10^6) were resuspended in 450 μl PBS and incubated with ferricytochrome c (100 μM, Sigma Chemical) in a total volume of 900 μl PBS in the presence or absence of mitoQ (1-20 μM) or other CoQ analogs (10-80 μM) for 15 min at 37ºC in spectrophotometric cells. The PMNs were stimulated with 100 nM PMA (Sigma Chemical) or 1 μM fMLP (Calbiochem) in a final reaction volume of 1.0 ml. Positive control samples were given SO dismutase (SOD; 10 μg/ml) just before the addition of PMA or fMLP. Absorbance at 550 nm was measured every 30 sec for up to 360 sec (peak response) for PMA and 180 sec for fMLP (peak response), and the change in absorbance (SO release) from PMNs was determined relative to time 0.

Cell Viability

Cell viability was determined by combining 0.5 ml of the samples from spectrophotometric analysis and 50 μl of 0.3% Trypan Blue. Then, 20 μl of the combined sample was placed on to a hemocytometer, and 100-150 cells were subsequently counted using microscopic analysis

Statistical Analysis

All data in the text and figures are presented as means ± S.E.M. The data were analyzed by analysis of variance using post hoc analysis with the Student-Newman-Keuls test. Probability values of ≤0.05 are considered to be statistically significant.

Results

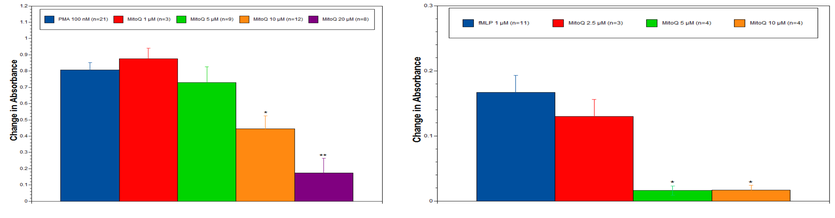

Figure 3. **The dose-dependent effects of MitoQ on PMA (100 nM) and fMLP (1μM)-induced SO release in PMNs**. The peak response in PMA -induced SO release (**left**). The peak response in fMLP -induced SO release (**right**). MitoQ (10μM) and mitoQ (20μM) significantly attenuated PMA-induced PMN SO release by 45% (*P<0.05) and 79% (**P<0.01) respectively compared to PMA. MitoQ (5μM) and mitoQ (10μM) significantly attenuated fMLP-induced PMN SO release by 90% (*P<0.05) and 90% (*P<0.05) respectively compared to fMLP. SOD served as a positive control inhibiting PMA-induced SO release by >90% (n=8; absorbance = 0.045 ± 0.005) and fMLP-induced SO release by >90% (n=9; absorbance = 0.010 ± 0.004).

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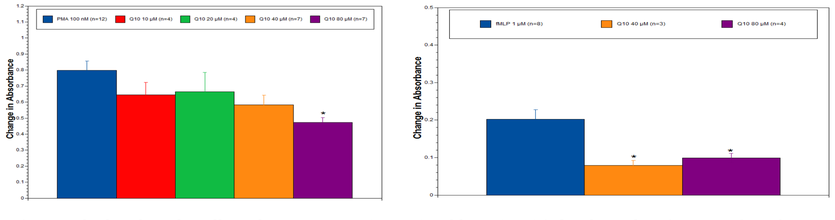

Figure 4. The dose-dependent effects of CoQ_{10} on PMA (100 nM) and fMLP (1μM)-induced SO release in PMNs. The peak response in PMA -induced SO release (**left**). The peak response in fMLP-induced SO release (**right**). CoQ_{10} (80μM) significantly attenuated PMA-induced PMN SO release by 41% (*P<0.05) compared to PMA. CoQ_{10} (40μM) and CoQ_{10} (80μM) significantly attenuated fMLP-induced PMN SO release by 61% (*P<0.05) and 51% (*P<0.05) respectively compared to fMLP.

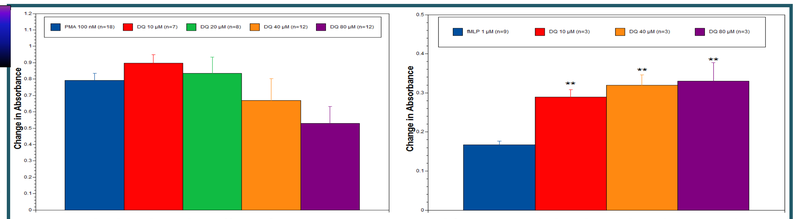

Figure 5. **The dose-dependent effects of DQ on PMA (100 nM) and fMLP (1μM)-induced SO release in PMNs**. The peak response in PMA -induced SO release (**left**). The peak response in fMLP -induced SO release (**right**). DQ did not significantly affect the PMA-induced SO release. DQ (10μM), DQ (40μM), and DQ (80μM) significantly augmented fMLP-induced PMN SO release by 73% (**P<0.01), 92% (**P<0.01), and 98% (**P<0.01) respectively compared to fMLP.

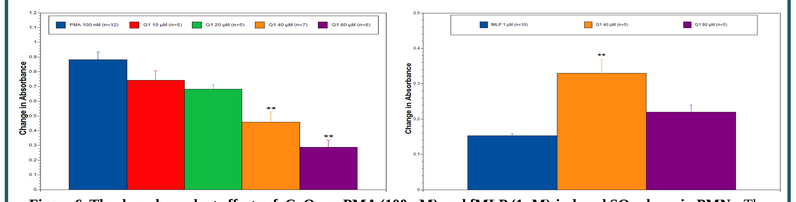

Figure 6. The dose-dependent effects of CoQ_1 on PMA (100 nM) and fMLP (1μM)-induced SO release in PMNs. The peak response in PMA -induced SO release (**left**). The peak response in fMLP -induced SO release (**right**). CoQ_1 (40μM) and CoQ_1 (80μM) significantly attenuated PMA-induced PMN SO release by 48% (**P<0.01) and 67% (**P<0.01) respectively compared to PMA. CoQ_1 (40μM) significantly augmented fMLP-induced PMN SO release by 84% (**P<0.01) compared to fMLP.

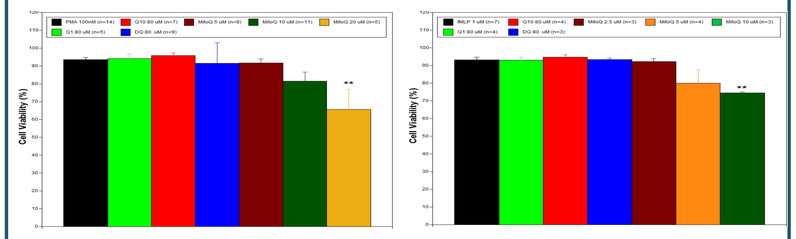

Figure 7. The Effects of CoQ analogs on Cell Viability in PMA (100 nM) and fMLP (1 μM)-induced SO release in PMNs. Cell Viability in PMA -induced SO release (**left**). Cell Viability in fMLP -induced SO release (**right**). MitoQ (20μM) significantly attenuated cell viability by 28% (**P<0.01) compared to PMA. MitoQ (10μM) significantly attenuated cell viability by 19% (*P<0.05) compared to fMLP.

Summary of Results

MitoQ and CoQ_{10} dose-dependently attenuated both PMA and fMLP-induced SO release in PMNs. DQ and CoQ_1 significantly augmented the fMLP-induced PMN SO release. Whereas, CoQ_1 dose-dependently significantly attenuated PMA-induced SO release, DQ showed a trend to decrease the PMA-induced response. CoQ_4 had no significant effect on PMA or fMLP-induced PMN SO release (*data not shown*). MitoQ significantly decreased cell viability in both PMA (20 μM) and fMLP (10 μM) stimulated PMNs. CoQ_1, CoQ_4, CoQ_{10}, and DQ had no significant effect on cell viability.

Conclusions

These results suggested that mitoQ exerted its antioxidant effects independent of the mitochondria and were related in part to cell viability. CoQ_{10} exerted its antioxidant effects independent of cell viability and suggests that the quinone group of the molecule was converted into its reduced form. DQ and CoQ_1 exerted pro-oxidant effects in fMLP stimulated PMNs perhaps by augmenting receptor-mediated signal transduction causing NADPH oxidase activation. Whereas, CoQ_1 exerted dose-dependent inhibition of PMA-induced PKC activation of NADPH oxidase .

References

1. Young, L.H., et al. *Gö 6983: A Fast Acting Protein Kinase C Inhibitor that Attenuates Myocardial Ischemia/Reperfusion Injury*. Cardiovascular Drug Reviews. **23**(3): p. 255-272.
2. Ondrasik, R., et al. *Cardioprotective effects of mitochondrial-targeted antioxidants in myocardial ischemia/reperfusion (I/R) injury.* Proceedings of the 23rd American Peptide Symposium. 2013. p. 64-65
3. Adlam, VJ, et al. *Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury*. FASEB Journal, 2005. **19**(9): p. 1088-1095.
4. Perkins, K. A., et al. *Myristoylation of Protein Kinase C Beta II/Zeta Peptide Inhibitors, or Caveolin-1 Peptide Facilitates Rapid Attenuation of Phorbol 12-Myristate 13-Acetate (PMA) or N-Formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP) Activated Leukocyte Superoxide Release*. Proceedings of the 22nd American Peptide Symposium. 2011. p. 288-289.