DINITROBENZENES STIMULATE ELECTRON FLUX WITHIN NEURONAL NITRIC OXIDE SYNTHASE IN THE ABSENCE OF CALMODULIN

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ABSTRACT

Efficient electron transfer and conversion of *L*-arginine to *L*-citrulline and nitric oxide ($NO^{\bullet}$) by neuronal nitric oxide synthase (nNOS) requires calmodulin (CaM) binding. The present study focused on electron transfer ability of resting state CaM-free nNOS in presence of dinitrobenzene isomers (DNBs). NADPH oxidation ($NADPH_{ox}$) and acetylated cytochrome-*c* reduction (AcCyt-c_{red}) catalyzed by nNOS and the CaM binding sequence-deficient nNOS reductase construct (nNOS-FP) were estimates of total electron flux and $O_2^{\bullet-}$ production, respectively. All the DNBs (*o*-, *m*-, *p*-) independently stimulated rates of $NADPH_{ox}$ by CaM-free nNOS and by nNOS-FP in isomer- and concentration-dependent manner. Blocking nNOS heme by imidazole or *L*-arginine did not affect CaM-free nNOS-catalyzed $NADPH_{ox}$ stimulated by DNBs. This stimulated electron flux by DNBs did not support $NO^{\bullet}$ formation by CaM-free nNOS. The DNBs, like FeCN, extract electrons from both FMN and FAD of the nNOS reductase domain. All three DNBs greatly stimulated nNOS and nNOS-FP catalyzed AcCyt-c_{red} that was significantly inhibited by SOD demonstrating $O_2^{\bullet-}$ formation. Thus, in presence of DNBs, resting-state CaM-deficient nNOS efficiently transfers electrons generating $O_2^{\bullet-}$, inferring that additional metabolic roles for nNOS exist that are not yet explored.

KEY WORDS: nNOS, NADPH, redox, superoxide

1. INTRODUCTION

Nitric oxide synthase isoforms consist of the flavin-containing reductase and heme-containing oxygenase domain[1] linked by a calmodulin (CaM) binding sequence[2]. The reductase domain is further made up of two sub-domains, one containing FMN and the other FAD[3]. The reductase domain of NOS accepts two electrons from NADPH and channels them individually through the flavins to the NOS heme that binds *L*-arginine, resulting in catalysis of a two step monoxygenation of *L*-arginine to nitric oxide ($NO^{\bullet}$) and *L*-citrulline[4]. The reductase domain of nNOS possesses 36% amino acid sequence identity and 58% homology to NADPH-cytochrome P450 oxidoreductase (CYPOR)[5]. Like CYPOR, NOS can catalyze the reduction of exogenous electron acceptors such as cytochrome-*c*, ferricyanide (FeCN), 2,6-dichlorophenolindo-phenol (DCIP)[6] and quinones[7]. However, unlike CYPOR, efficient electron transfer to and through nNOS is governed by the binding of

Ca^{2+}/CaM to nNOS[8] making nNOS the most highly regulated enzymes known. The present study explored mechanisms of electron transfer through resting state nNOS and the nNOS reductase construct not containing the CaM binding domain (aa. 743-1429) (nNOS-FP)[9] in the presence of three specific DNBs, *o*-, *m*-, and *p*-, used as model compounds earlier reported to redox cycle[10]. This report illustrates the much higher electron transfer capability of the resting state CaM-free nNOS enzyme that results in formation of $O_2^{\bullet-}$ in the presence of DNBs.

2. MATERIALS AND METHODS

2.1. ***Caution:*** *DNB isomers are highly toxic and potentially mutagenic and should be handled in a fume hood by experienced laboratory personnel.*

2.2. Materials: 2',5'-ADP Sepharose 4B was purchased from Amersham-Pharmacia Biotech (Piscataway, NJ). Equine heart ferricytochrome-*c*, (*6R*)-5,6,7,8-tetrahydro-*L*-biopterin (BH_4), *L*-arginine•HCl, imidazole•HCl, chloramphenicol, bovine brain CaM, $CaCl_2$•$2H_2O$, KH_2PO_4, NaCl, freshly distilled acetic anhydride, Dowex 50W-X8, dithiothretol (DTT), *β*-nicotinamide adenine dinucleotide phosphate (*β*-NADPH; reduced form), yeast adenosine 2-monophosphate, Cu/Zn superoxide dismutase (SOD, bovine erythrocytes; 4770 units/mg), catalase (bovine liver, 9360 units/mg), riboflavin, Tris•HCl, HEPES, adenosine triphosphate, phenylmethylsulfonyl flouride, lysozyme, leupeptin and DMSO were from Sigma (St. Louis, MO). [^{14}C]-*L*-arginine (300mCi/mMol) was from Perkin-Elmer, (Shelton, CT). Sodium dithionite, *o*-DNB and *p*-DNB were from Aldrich Chemical Co. (Milwaukee, WI). Ampicillin, *m*-DNB and pepstatin were from Fluka (Switzerland). Na_2HPO_4 was from Fisher Biotech (Fairlawn, NJ) and 2-mercaptoethanol was from Fisher Scientific (Freeway, NJ). 5-Aminolevulinic acid and isopropyl-beta-D-thiogalactopyranoside (IPTG) were from Research Products International (Mt. Prospect, IL). Glycerol, tryptone and yeast extract were from EMD Bioscience (Germany) and Ni-NTA superflow resin was purchased from Quiagen.

2.3. Enzyme preparation: Recombinant rat cerebellar nNOS was prepared as described[11]. The isolated nNOS dimer had a specific turnover of 36 min^{-1}. A His-tagged nNOS flavoprotein construct (aa. 743-1429; nNOS-FP) was prepared[9] and the peak fraction of eluate from the Ni-NTA column was used. Specific cytochrome-*c* reduction activity for the nNOS-FP construct was 29 min^{-1}.

2.4. NADPH oxidation assay: The NADPH assay mix consisted of 100 µM NADPH in 1 ml 50 mM HEPES (pH 7.6). The individual DNB isomers were titrated depending on pre-determined concentrations. Imidazole•HCl (1 mM) or *L*-arginine•HCl (100 µM) were added to specific reactions to block O_2 reduction by nNOS[12]. Reactions were initiated by adding 20 pmol nNOS or nNOS-FP. Positive controls contained 20 nM CaM with 400 µM $CaCl_2$. Reference cuvettes lacked enzyme. Reactions were monitored at 340 nm (Shimadzu 2401-PC dual-beam spectrophotometer) for 1 min at 23°C, steady state kinetics[13] calculated using $\varepsilon = 6.22\ mM^{-1}cm^{-1}$.

2.5. Cytochrome-*c* reduction assay: The assay consisted of 40 µM cytochrome-*c* and 100 µM NADPH in 1 ml 50 mM HEPES (pH 7.6). To initiate reactions, 5 pmol nNOS or nNOS-FP was added. Calmodulin and $CaCl_2$ were added to specific reactions to determine the CaM stimulation. Reactions were executed for 1 minute at 23°C and monitored at 550 nm, reaction rates[14] calculated using $\varepsilon = 21\ mM^{-1}cm^{-1}$.

2.6. [^{14}C]-*L*-arginine to [^{14}C]-*L*-citrulline conversion: The rate of [^{14}C]-*L*-arginine to [^{14}C]-*L*-citrulline conversion was used to measure nNOS activity[8]. Reactions consisted of 100 µM NADPH, 20 µM *L*-arginine containing 0.5 µCi radiolabelled *L*-arginine (300mCi/mMol) in 0.25 ml 50 mM HEPES/KOH (pH 7.6). Individual DNB isomers at specific concentrations were added as pre-determined. Reactions were initiated with 5 pmol nNOS, executed for 5 min at 23° C and stopped with ice-cold solution containing 1 mM *L*-citrulline and 10 mM EDTA in 100 mM HEPES (pH 5.5). Negative controls lacked enzyme. [^{14}C]-*L*-citrulline was eluted from resin columns and radioactivity measured by liquid scintillation. Positive controls consisted of all ingredients with Ca^{2+}/CaM.

2.7. Acetylated cytochrome-*c* reduction assay: Reduction of acetylated cytochrome-*c* was used to estimate $O_2^{\bullet-}$ production. Horse heart ferricytochrome-*c* was acetylated with acetic anhydride, substituted for succinic anhydride[15]. Acetylated cytochrome-*c* reduction assays were performed as described[16]. The assay mix consisted of 40 µM acetylated cytochrome-*c* and 100 µM NADPH in 1 ml 50 mM HEPES (pH 7.6). The individual DNB isomers were titrated depending on pre-determined concentrations. Reactions were initiated by addition of 20 pmol nNOS or nNOS-FP and monitored at 550 nm for 1 min at 23° C, steady state reaction kinetics[14] were calculated using ε = 21 $mM^{-1}cm^{-1}$. Cu/Zn SOD was titrated into specific reactions up to 100U/reaction.

2.8. Protein determination of holo-nNOS and nNOS-FP constructs: The nNOS protein content was estimated from dithionite-reduced CO difference spectrum generated from 30 µl of enzyme aliquot. To the diluted aliquot, sodium dithionite (5-10 grains) was added and the reduced solution was divided equally into sample and reference cuvettes. After obtaining baseline, the sample cuvette was exposed to 15-20 CO bubbles and spectrum of CO-bound nNOS was recorded between 400 and 700 nm. Protein concentration was determined based on heme content using ε = 100 $mM^{-1}cm^{-1}$ for a Δ*A* of 444 nm minus 470 nm[17]. Protein concentration of nNOS-FP (aa. 743-1429) was estimated against a PBS reference using ε = 21 $mM^{-1}cm^{-1}$ at 455nm for total flavin content[18].

2.9. Statistics: Student's unpaired t-test was used to obtain statistical significance ($p < 0.05$). Unless otherwise noted, data are mean ± SEM obtained from 3 independent experiments each performed in triplicate.

3. RESULTS

Figure 1 shows the DNB isomer- and concentration-dependent increase in NADPH oxidation rates catalyzed by CaM-free nNOS in different heme-states (Fig. 1A: holo nNOS; Fig. 1B: imidazole-bound nNOS; Fig. 1C: *L*-arginine-bound nNOS). The most potent isomer stimulating NADPH oxidation was *p*-DNB, followed respectively by *o*-DNB and *m*-DNB. The presence of *L*-arginine or imidazole had no effect on DNB stimulated CaM-free nNOS catalyzed NADPH oxidation rates. Similar trends in DNB stimulation of NADPH oxidation catalyzed by nNOS-FP were observed (Fig. 1D), although the turnover rates were almost half of those observed with holo-nNOS. The presence of Ca^{2+}/CaM further stimulated (~3-fold, Table 1) imidazole-bound nNOS-catalyzed NADPH oxidation that was already stimulated by the DNB isomers. CaM-free nNOS in the presence of DNB isomers was unable to catalyze conversion of *L*-arginine to *L*-citrulline and $NO^{\bullet}$. The CaM-bound nNOS positive control

catalyzed [^{14}C] *L*-citrulline formation at a rate of 15 ± 0.2 min^{-1} compared to virtual absence of product formation with CaM-free nNOS even in the presence of any of the DNB isomers (Fig. 2). Under conditions similar to the NADPH oxidation assay, acetylated cytochrome-*c* reduction catalyzed by both nNOS (Fig. 3A) and nNOS-FP (Fig. 3B) was significantly stimulated in presence of DNB isomers compared to vehicle controls and this stimulation was significantly inhibited by SOD, indicating $O_2^{\bullet}$ formation. This maximum stimulation was in presence of *p*-DNB, followed by *o*-DNB and *m*-DNB, respectively.

DISCUSSION

The current study provides insights into the electron transfer properties of resting state nNOS in the absence of Ca^{2+}/CaM and describes novel results supporting its electron transfer capability in the absence of Ca^{2+}/CaM but only in the presence of DNB isomers. The NADPH oxidation assay represents an estimate of the total electron flux in the system and this stimulation of nNOS-catalyzed NADPH oxidation by DNB isomers was dependent on both the structure and the concentration. The study of structure is important as it exemplifies that easily reducible structures favor more rapid electron transfer. The stimulation of nNOS-FP-catalyzed NADPH oxidation in the presence of DNB isomers supports involvement of the flavoprotein domain of nNOS in stimulating electron flux occurring in the presence of DNBs. These results suggests that the nNOS reductase domain is sufficient to mediate electron transfer to the DNB isomers and the stimulation of electron flow to and through nNOS is not dependent on Ca^{2+}/CaM stimulation but rather depends on presence of chemicals that can undergo reduction. In the presence of DNB isomers, CaM stimulated the rate of imidazole-bound nNOS-catalyzed NADPH oxidation by a factor of ~3, similar to stimulation of FeCN reduction in the presence of CaM[19]. Thus similar to FeCN, DNBs may extract electrons from both FAD and FMN of the nNOS reductase domain. None of the DNB isomers that stimulated CaM-free nNOS-catalyzed electron flux catalyzed the conversion of *L*-arginine to *L*-citrulline. The DNB isomers thus cannot substitute Ca^{2+}/CaM for nNOS activity. The DNB reduction taking place is similar to many of the electron transfer reactions catalyzed by CYPOR[20] that may also be catalyzed by nNOS. Neuronal NOS thus appears to have the ability to reduce specific chemical compounds and further studies are warranted in order to study the metabolizing potential of nNOS.

Under conditions similar to the NADPH oxidation assay, the presence of DNBs stimulated acetylated cytochrome-*c* reduction rates by nNOS and nNOS-FP in a SOD-dependent manner. The SOD-inhibitable component in this reaction translates into $O_2^{\bullet}$ production. The DNB isomer structure-dependent differential stimulation in electron flux clearly illustrates the ease of 1 electron reduction of *p*-DNB compared to *o*- and *m*-DNB, catalyzed by CaM-free nNOS and nNOS-FP. *p*-DNB readily accepts an electron from nNOS and nNOS-FP and donates it to molecular O_2. This rate of single electron loss to O_2 is the process of DNB reoxidation. Thus, reoxidation is a passive process and the stability of the nitro-anion radical formed upon a DNB isomer receiving an electron will necessarily be a factor in determining its rate of reoxidation. The 1 electron-reduced nitroxyl radical in the case of *p*-DNB is a very reactive species and rapidly donates its electron to O_2 forming

$O_2^{\bullet-}$ and reoxidizing to *p*-DNB. The 1 electron reduced *o*- and *m*-DNB nitroxyl radicals appear to be more stable than *p*-DNB, apparent from the lower rates of $O_2^{\bullet-}$ formed in the presence of *o*- and *m*-DNB, even when used at concentrations higher than *p*-DNB. Overall these results provide strong evidence for CaM-free nNOS catalyzed $O_2^{\bullet-}$ formation from O_2 in the presence of DNB isomers. This initiation and persistent flow of electrons is principally governed by diflavin-containing reductase domains within nNOS. A schematic presentation of the electron flow through this system is shown in Fig. 4, where electrons originating from NADPH travel through the nNOS flavins and DNB isomers and resulting in the reduction of oxygen to superoxide.

CONCLUSION

In conclusion our data suggest that 1) in the absence of CaM, DNB isomer- and concentration-dependent stimulation of electron flux through nNOS surpasses the electron flux observed during the active $NO^{\bullet}$ synthesis by CaM-bound nNOS; 2) the promotion of electron transfer to and through nNOS is dependent on the presence of redox active chemicals and not on Ca^{2+}/CaM; and 3) redox cycling of the DNB isomers catalyzed by resting state CaM-free nNOS produces $O_2^{\bullet-}$ and can lead to depletion of cellular energy stores by consuming NADPH. Additional biological functions that nNOS may possess have not yet been characterized.

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Table 1: nNOS catalyzed DNB isomers stimulated NADPH oxidation rate enhanced by CaM.

Imidazole-bound nNOS alone catalyzed NADPH oxidation rate stimulated by DNBs was further enhanced by the presence of Ca^{2+}/CaM by a factor of ~3, which is comparable to that reported earlier in the presence of FeCN[18]. The fold stimulation values are obtained from mean V_{max} min^{-1} from 2 independent experiments each performed in triplicate.

Experimental condition	NADPH Oxidation (V_{max} min^{-1})	Fold stimulation $\frac{(NADPH_{ox} \text{ with CaM})}{(NADPH_{ox})}$
16 µM *p*-DNB	75	3.5
16 µM *p*-DNB + CaM	263	
40 µM *o*-DNB	40	2.9
40 µM *o*-DNB + CaM	117	
160 µM *m*-DNB	34	2.9
160 µM *m*-DNB + CaM	97	

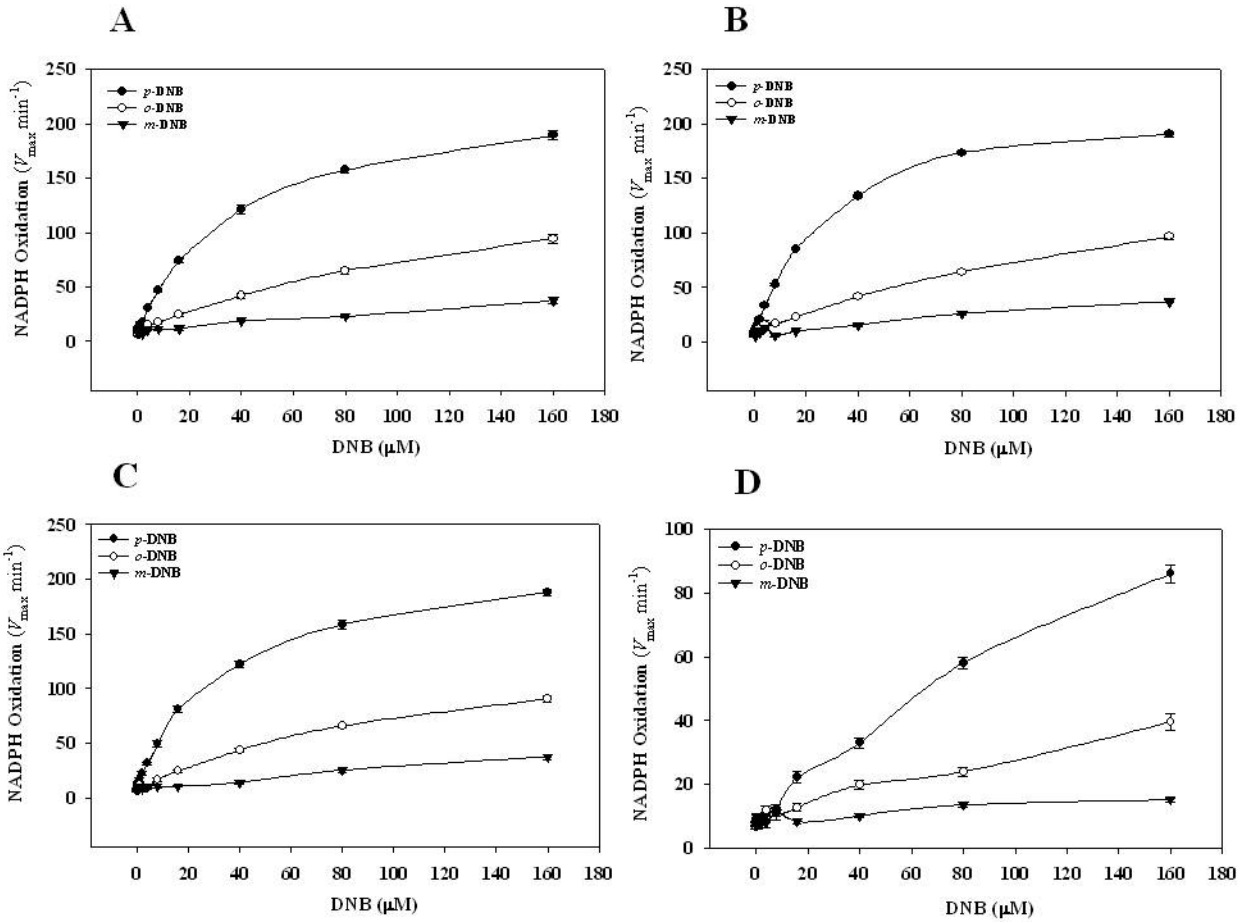

Figure 1. NADPH oxidation catalyzed by CaM-free nNOS and nNOS-FP in the presence of DNBs.

Turnover rates (V_{max} min^{-1}) for DNB isomer- and concentration-dependent stimulation of NADPH oxidation catalyzed by: (A) CaM-free nNOS; (B) imidazole-bound CaM-free nNOS; (C) *L*-arginine-bound CaM-free nNOS; and (D) nNOS-FP. The maximum stimulation was observed in the presence of *p*-DNB followed by *o*-DNB and *m*-DNB.

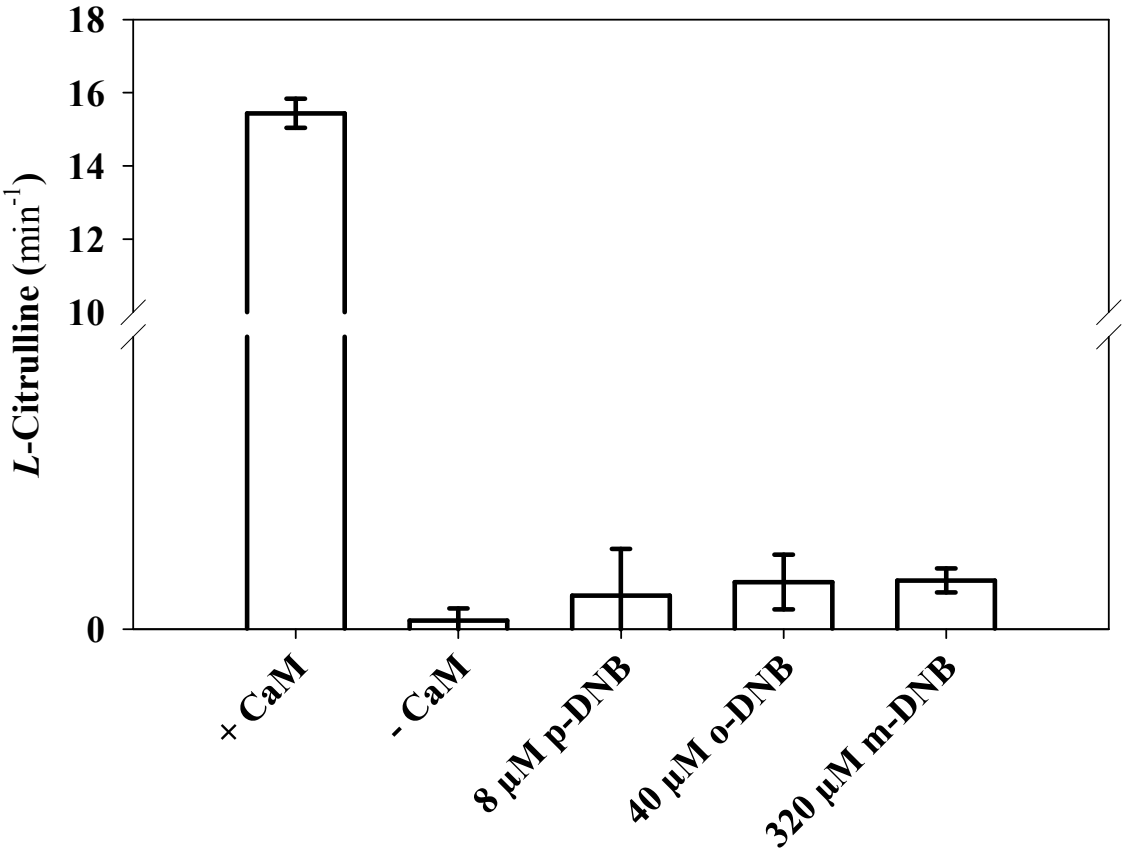

Figure 2. Formation of *L*-citrulline by CaM-free nNOS in the presence of DNBs.

The CaM-free nNOS is unable to produce *L*-citrulline with efficient electron flux in presence of DNB isomers. The *L*-citrulline formation by nNOS is observed only in the presence of CaM. In the absence of CaM and presence of the DNB isomers, *L*-citrulline formation was not observed. Data are mean ± SE from 3 independent experiments each performed in duplicate.

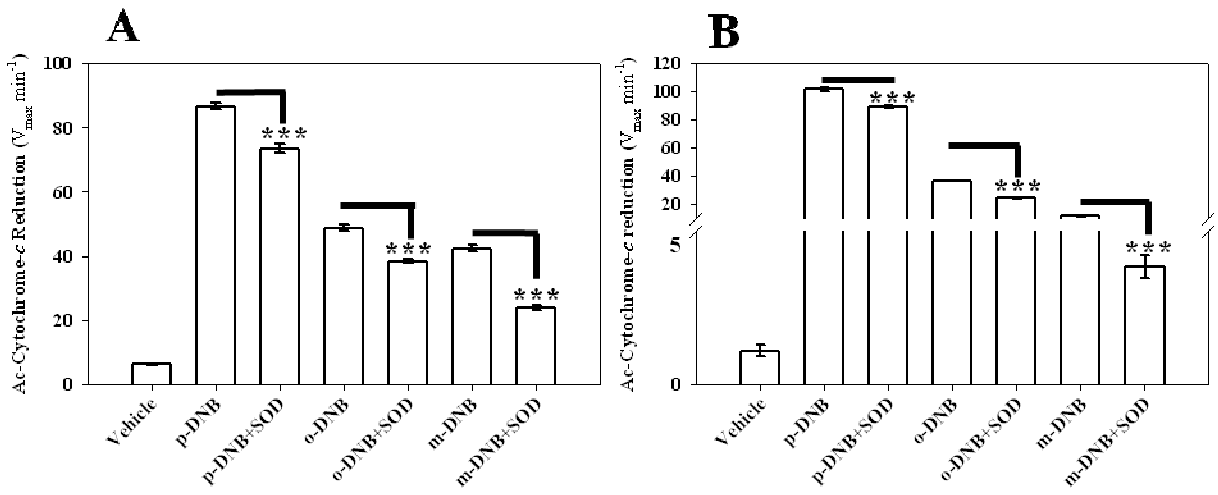

Figure 3. Acetylated cytochrome-c reduction by CaM-free nNOS and nNOS-FP in the presence of DNB isomers.

Significant stimulation of acetylated cytochrome-*c* (AcCyt-*c*) reduction (V_{max} min^{-1}) in the presence of DNB isomers catalyzed by: (A) CaM-free nNOS holoenzyme and (B) nNOS-FP construct. The reactions catalyzed by CaM-free nNOS were carried out in the presence of 8 µM *p*-DNB, 40 µM *o*-DNB and 160 µM *m*-DNB respectively, and those by nNOS FP were in the presence of 80 µM *p*-DNB and 160 µM *o*-DNB and *m*-DNB respectively. Addition of SOD (100U) isomers significantly inhibited (*** = $p < 0.001$) both the nNOS- and nNOS-FP-catalyzed acetylated cytochrome-*c* reduction rates.

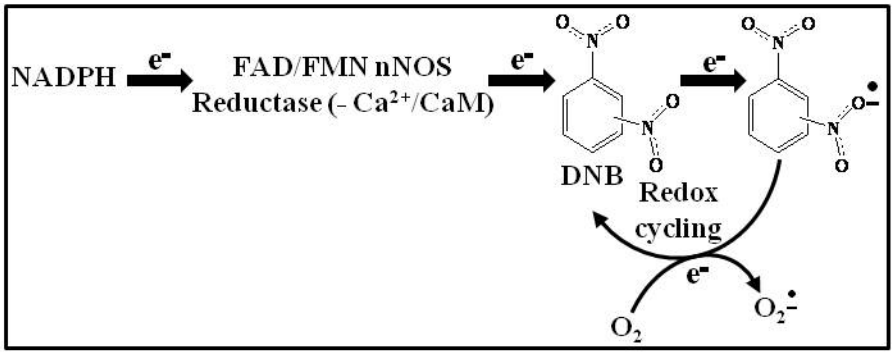

Figure 4: Electron flow to and through nNOS reductase in the presence of DNBs. Schematic showing the electron flow initiating from NADPH, flowing to and through the nNOS reductase, reducing the DNBs that redox cycle and ultimately reduce O_2 to $O_2^{\bullet-}$