Ceramide-induced disruption of endothelial nitric oxide synthase dimerization in bovine aortic endothelial cells (BAECs) is not secondary to peroxynitrite formation

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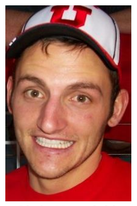

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BACKGROUND AND PRELIMINARY DATA

An estimated 23.6 million individuals in the United States have diabetes and of those 90-95% have type II diabetes. Cardiovascular complications (e.g., hypertension and vascular dysfunction) are four-fold more prevalent in patients with type II diabetes.[1]

The mechanism(s) responsible for increased susceptibility of type II diabetics to cardiovascular complications is unclear.

In a previous study, mice that consumed high-fat (HF) vs. standard (CON) chow for 10-14 weeks exhibited: systemic disturbances characteristic of the metabolic syndrome; vascular dysfunction; and hypertension.[2] Because free fatty acids (FFAs) were elevated three-fold in those HF mice, we investigated whether the fat derived metabolite ceramide might contribute to cardiovascular complications.

To do so, mice consumed HF chow and were treated concurrently with the ceramide biosynthesis inhibitor myriocin or vehicle. Cardiovascular (e.g., hypertension and vascular dysfunction) and metabolic (e.g., impaired glucose tolerance and dyslipidemia) complications did not develop in myriocin vs. vehicle-treated HF mice.[3] Importantly, subsequent in vitro experiments using pharmacological and genetic approaches to inhibit ceramide biosynthesis showed this sphingolipid impairs endothelium-dependent function in a tissue autonomous manner.[4]

To gain insight into mechanisms responsible for ceramide-induced endothelial dysfunction, BAECs were incubated with palmitate to precipitate endogenous ceramide biosynthesis. Results are shown in Fig. 1 – Panels A-C.

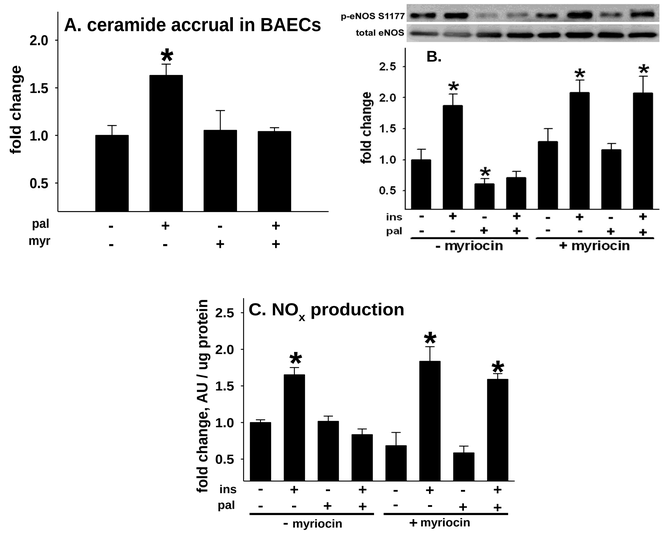

FIG 1. A. Palmitate (pal, 3 h x 0.5 mM) -induced ceramide accrual in BAECs is prevented by co-incubation with 10 µM myriocin (myr, *p<0.05 vs. all). **B and C.** BAECs were incubated for 3 h pal myr 100 nM insulin (ins). Basal and ins-stimulated p-eNOS to total eNOS S1177 (**B**); and ins-stimulated nitric oxide (NO) production (estimated by nitrate + nitrite in the cellular media, NOx; **C**) are abolished by pal, but are restored by co-incubation with myr. [*p<0.05 vs. (-ins) (-pal)(-myr)]. **Thus, pal-induced reductions in p-eNOS and NO production occur in a ceramide-dependent manner.**

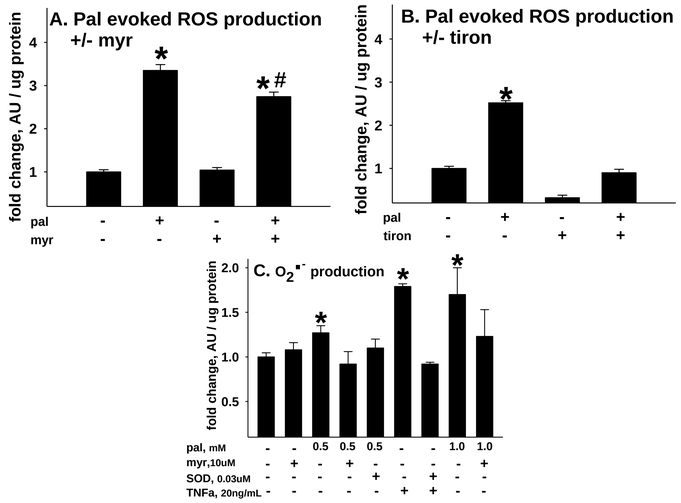

FIG 2. BAECs were incubated for 3 h pal myr **(A)** or the intracellular superoxide anion ($O_2^{\cdot-}$) scavenger tiron **(B).** For the last 30-min BAECs were treated with the fluorescent dye 2',7'–dichlorofluoroscein diacetate (DCF). DCF detects hydrogen peroxide (H_2O_2; see Fig 3) and is an estimate of overall reactive oxygen species (ROS) generation. **A** Pal-induced ROS generation by BAECs was blunted after co-incubation with myr. **B** Pal-induced ROS generation was abolished by co-incubation with tiron. **C** Direct assessment of $O_2^{\cdot-}$ accumulation in BAECs was performed using EPR. Pal-induced $O_2^{\cdot-}$ accumulation was abolished by co-incubation (+) with either myr or SOD (n=8+ per treatment). TNFa (Tumor Necrosis Factor Alpha) was used as a positive control. TNFa-induced $O_2^{\cdot-}$ production was abolished in the presence of SOD. **Thus, pal-induced ceramide accumulation increases ROS and $O_2^{\cdot-}$.**

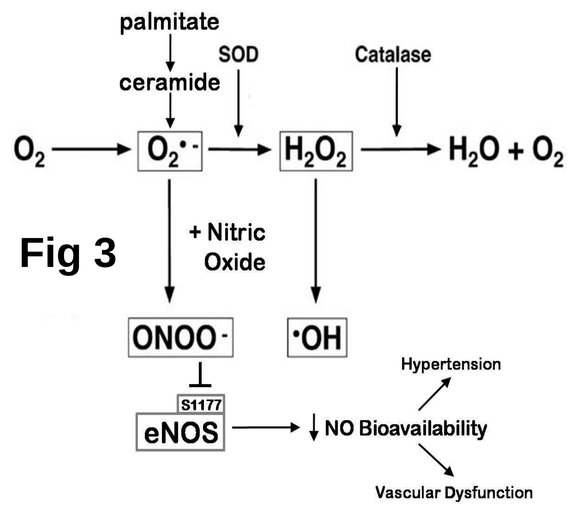

FIG 3. Working hypothesis. FFAs might precipitate hypertension and vascular dysfunction by lowering NO bioavailability. NO bioavailability can result from decreased NO synthesis [e.g., by impaired endothelial NO synthase (eNOS) enzyme structure/function] and/or by increased NO destruction (e.g., via elevated oxidant load). Preliminary data indicate that pal-induced ceramide biosynthesis increases ROS production in general (Fig 2 A,B), and $O_2^{\cdot-}$ production in particular (Fig 2 C). If endogenous antioxidant mechanisms are effective then $O_2^{\cdot-}$ is rapidly dismuted to H_2O_2 via superoxide dismutase (SOD), and H_2O_2 is converted to H_2O and O_2 via catalase. If $O_2^{\cdot-}$ generation overwhelms cellular antioxidant mechanisms then $O_2^{\cdot-}$ is available to react with NO to form peroxynitrite (ONOO-). Evidence exists that ONOO- disrupts the eNOS monomer:dimer ratio. Since eNOS dimers must be present for optimal eNOS enzymatic activity, ONOO- formation might disrupt eNOS structure/function and precipitate lower NO bioavailability. **We hypothesized that ceramide-induced $O_2^{\cdot-}$ production results in peroxynitrite formation to an extent that disrupts eNOS monomer: dimer formation.**

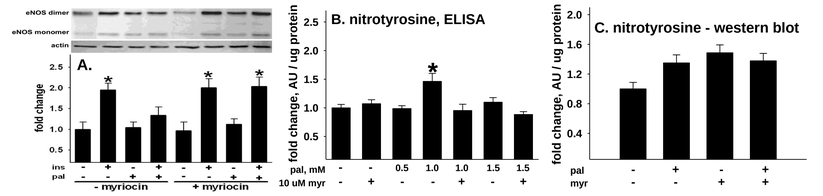

FIG 4. A. BAECs were incubated for 3 h pal myr and were (+) or were not (-) stimulated with ins. Pal incubation prevented ins-stimulated eNOS monomer:dimer ratio formation but the response was restored by co-incubation with myr. [*p<0.05 vs. (–ins)(-pal)(-myr)]. Results were similar using VEGF as the agonist (data not shown). Next, we sought to determine whether ceramide-induced disruption of the eNOS monomer:dimer formation was secondary to ONOO- formation. ONOO- is very unstable and difficult to quantify. An accepted "footprint" to estimate ONOO- formation is nitrotyrosine. 0.5 mM pal did not alter nitrotyrosine formation assessed using ELISA **(B),** western blot **(C),** or immunostaining (data not shown). **Thus, 500 uM pal disrupts the eNOS monomer:dimer ratio in a ceramide-dependent manner via mechanisms that are not secondary to ONOO- formation.**

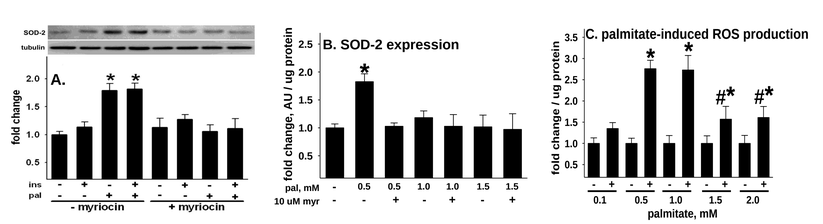

FIG 5. It was unclear to us why 500 uM pal generated $O_2^{\cdot-}$ (Fig 2C) but did not precipitate ONOO- formation (Fig. 4 B,C). One explanation is that SOD capably dismuted $O_2^{\cdot-}$ to H_2O_2, and catalase converted H_2O_2 to H_2O and O_2 (Fig 3). To test this, SOD expression was assessed in BAECs treated ins pal myr. **A.** Pal increased SOD expression in the absence and presence of ins. In both cases this response was prevented by myr. Thus, pal-induced $O_2^{\cdot-}$ generation was capably dismuted to H_2O_2 via increased SOD expression.

If increased SOD expression explains why ONOO- formation did not occur in Fig 4 (B,C), it follows that ONOO- formation should occur if SOD expression is overwhelmed. To test this, BAECs were incubated with a higher dose of pal (i.e.,1.0 mM; Figs 2C, 4B). Compared to 500 uM pal, $O_2^{\cdot-}$ generation is greater (Fig 2C), SOD expression and ROS production do not increase further **(Fig 5B,C)**, and ONOO- formation does indeed occur (Fig 4B). **Thus, 500 uM pal-induced reductions in NO bioavailability are not secondary to $O_2^{\cdot-}$ mediated increases in ONOO- formation. This is because endogenous intracellular antioxidant mechanisms capably convert 500 uM pal-induced $O_2^{\cdot-}$ to H_2O_2.**

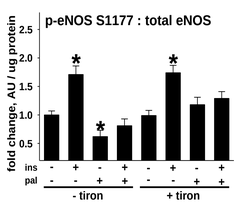

FIG 6. If 500 uM pal-induced reductions in basal and ins-stimulated p-eNOS are not secondary to $O_2^{\cdot-}$ production and subsequent ONOO- formation, it follows that tiron should not be effective in restoring the response. FIG 6 indicates that basal and ins-stimulated reductions in p-eNOS are not restored by co-incubation with tiron. **Thus, pal-induced reductions in basal and ins-stimulated p-eNOS S1177 occur in a manner that is independent from $O_2^{\cdot-}$ production.**

CONCLUSION

While pal-induced ceramide accumulation disrupts the eNOS monomer : dimer ratio, the mechanism is not secondary to $O_2^{\cdot-}$ -induced ONOO- formation and remains to be determined.

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