Freeman BA, Khoo NK. Fatty acid nitroalkenes ameliorate glucose intolerance and pulmonary hypertension in high fat diet-induced obesity. *Cardiovasc Res* 2014;**101**:352–363.
2. Tsikas D, Zoerner AA, Mitschke A, Gutzki FM. Nitro-fatty acids occur in human plasma in the picomolar range: a targeted nitro-lipidomics GC-MS/MS study. *Lipids* 2009;**44**:855–865.
3. Trettin A, Böhmer A, Zoerner AA, Gutzki FM, Jordan J, Tsikas D. GC-MS/MS and LC-MS/MS studies on unlabelled and deuterium labelled oleic acid (C18:1) reactions with peroxynitrite ($ONOO^-$) in buffer and hemolysate support the pM/nM-range of nitro-oleic acids in human plasma. *J Chromatogr B* 2014; http://dx.doi.org/10.1016/j.jchromb.2014.01.016.
4. Liu Y, Jia Z, Liu S, Downton M, Liu G, Du Y, Yang T. Combined losartan and nitro-oleic acid remarkably improves diabetic nephropathy in mice. *Am J Physiol Renal Physiol* 2013;**305**:F1555–F1562.
5. Zhang J, Villacorta L, Chang L, Fan Z, Hamblin M, Zhu T, Chen CS, Cole MP, Schopfer FJ, Deng CX, Garcia-Barrio MT, Feng YH, Freeman BA, Chen YE. Nitro-oleic acid inhibits angiotensin II-induced hypertension. *Circ Res* 2010;**107**:540–548.
6. Rudolph TK, Rudolph V, Edreira MM, Cole MP, Bonacci G, Schopfer FJ, Woodcock SR, Franek A, Pekarova M, Khoo NK, Hasty AH, Baldus S, Freeman BA. Nitro-fatty acids reduce atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2010;**30**:938–945.
7. Awwad K, Steinbrink SD, Frömel T, Lill N, Isaak J, Häfner AK, Roos J, Hofmann B, Heide H, Geisslinger G, Steinhilber D, Freeman B, Maier TJ, Fleming I. Electrophilic fatty acid species inhibit 5-lipoxygenase and attenuate sepsis-induced pulmonary inflammation. *Antioxid Redox Signal* 2013; doi:10.1089/ars.2013.5473.
8. Rudolph V, Schopfer FJ, Khoo NK, Rudolph TK, Cole MP, Woodcock SR, Bonacci G, Groeger AL, Golin-Bisello F, Chen CS, Baker PR, Freeman BA. Nitro-fatty acid metabolome: saturation, desaturation, beta-oxidation, and protein adduction. *J Biol Chem* 2009;**284**:1461–1473.

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Nitro-oleic acid and epoxyoleic acid are not altered in obesity and Type 2 diabetes: reply

We appreciate the interest in our recent study of the *in vivo* signalling actions of electrophilic fatty acid nitroalkenes,[1] including the commentary from Tsikas *et al.* This study is based on a high-fat diet (HFD)-induced obesity murine model that displays the canonical features of metabolic syndrome, including glucose tolerance, vascular dysfunction, increased inflammation, adipokine dysfunction, and most notably development of pulmonary hypertension. We tested the pharmacological actions of synthetic 10-nitro-octadec-9-enoic acid (OA-NO_2) and reported potent protective actions of OA-NO_2 in this murine model of HFD-induced obesity and pulmonary hypertension. First and foremost, we stress that under no circumstance did we state or imply the existence of any link between obesity and decreased OA-NO_2 biosynthesis. This is an incorrect and misleading interpretation of the data presented in our manuscript.

The letter from Tsikas *et al.* presents new data indicating no difference in endogenous levels of OA-NO_2 in lean, obese individuals, and obese Type 2 diabetics. We read this letter with interest as multiple groups including our own are striving to determine the mechanism(s) of endogenous nitro-fatty acid (NO_2-FA) formation, to define the metabolism of these species and their products, and to understand how these mediators modulate signalling pathways *in vivo*. While the data provided by Tsikas *et al.* are intriguing, little analytical information was provided which greatly limits interpretation. Considering these concerns, we view this work should be judged on its merit and in its entirety in a peer-reviewed process.

In further clarification of points raised by Tsikas *et al.* regarding our present report, we disagree with Tsikas *et al.*'s opinion that OA-NO_2 is a 'weak' drug. This pharmacological term was used inappropriately, and in the peer-reviewed literature our group and others have reported that (i) the nitroalkene substituent confers electrophilic reactivity to a broad array of molecules and (ii) low concentrations of OA-NO_2 exert significant pleiotropic anti-inflammatory actions in a wide range of animal models of metabolic and inflammatory diseases.[2] Moreover, we observed no signs of toxicity at the concentration used (8 mg/kg/day) in the HFD-fed mice. These concentrations are significantly less than those considered safe in rodent, canine, and primate models, from recent preclinical pharmacokinetic and toxicology studies.

Notably, Tsikas *et al.* claim that OA-NO_2 has an inert vinyl functionality[3] and suggest a minor or no reactivity towards nucleophiles such as cysteine. Reaction kinetics studies refute this unsubstantiated view and support that OA-NO_2 rapidly reacts with biological thiols ($k_{GSH} = 183 \pm 6\ M^{-1}\ s^{-1}$ compared with $\sim 1\ M^{-1}\ s^{-1}$ for 4-hydroxy-2-nonenal and 15-deoxy-Δ12,14-prostaglandin J_2).[4] For example, the concentration of reduced Cys34 in the albumin component of human serum is 0.45 mM, and OA-NO_2 has an equilibrium constant for the reaction with cysteine (K_d) of 7.5×10^{-6} M.[5] These physical properties are consistent with the observation that OA-NO_2 rapidly forms protein adducts via Michael addition when added to plasma samples and when present in the vascular compartment. In this regard, we express concern that the analytical method used by Tsikas *et al.*[3,6] does not consider this Michael addition reaction, additional nitroalkene reactions, and acidic nitration, all of which can lead to inaccuracies in the determination of NO_2-FA species. Overall, we view that the quantification of reactive (thus metastable) fatty acid nitroalkenes should be reported in a way that benefits the scientific community, with documented methods and controls and not via methodologically incomplete letters.

In summary, the goal of our study was to test the therapeutic efficacy of the exemplary fatty acid nitroalkene OA-NO_2 in a model of HFD-induced obesity and pulmonary hypertension. Ongoing studies explore endogenous formation of this and other more predominant NO_2-FA species, their protein adducts, complex lipid esterified species and their metabolites—in both murine models and clinical samples. We are grateful that other laboratories are also pursuing a fruitful area of cell signalling and pharmacological research.

References

1. Kelley EE, Baust J, Bonacci G, Golin-Bisello F, Devlin JE, St Croix CM, Watkins SC, Gor S, Cantu-Medellin N, Weidert ER, Frisbee JC, Gladwin MT, Champion HC, Freeman BA, Khoo NK. Fatty acid nitroalkenes ameliorate glucose intolerance and pulmonary hypertension in high fat diet-induced obesity. *Cardiovasc Res* 2014;**101**:352–363.
2. Delmastro-Greenwood M, Freeman BA, Gelhaus Wendell S. Redox-dependent anti-inflammatory signaling actions of unsaturated fatty acids. *Ann Rev Physiol* 2014;**76**:8.1–8.27.
3. Tsikas D, Zoerner A, Mitschke A, Gutzki F-M. Nitro-fatty acids occur in human plasma in the picomolar range: a targeted nitro-lipidomics GC–MS/MS study. *Lipids* 2009;**44**:855–865.
4. Baker LM, Baker PR, Golin-Bisello F, Schopfer FJ, Fink M, Woodcock SR, Branchaud BP, Radi R, Freeman BA. Nitro-fatty acid reaction with glutathione and cysteine. Kinetic analysis of thiol alkylation by a Michael addition reaction. *J Biol Chem* 2007;**282**:31085–31093.
5. Salvatore SR, Vitturi DA, Baker PRS, Bonacci G, Koenitzer JR, Woodcock SR, Freeman BA, Schopfer FJ. Characterization and quantification of endogenous fatty acid nitroalkene metabolites in human urine. *J Lipid Res* 2013;**54**:1998–2009.
6. Tsikas D, Zoerner A, Mitschke A, Homsi Y, Gutzki F-M, Jordan J. Specific GC–MS/MS stable-isotope dilution methodology for free 9- and 10-nitro-oleic acid in human plasma challenges previous LC–MS/MS reports. *J Chromatogr B* 2009;**877**:2895–2908.

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