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UVA Irradiation of Human Skin Vasodilates Arterial Vasculature and Lowers Blood Pressure Independently of Nitric Oxide Synthase.

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Short title: UVA mobilises cutaneous NO to lower BP

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Abbreviations:

Blood Pressure :BP Cardiovascular Disease: CVD. Diaminofluorescein2 Diacetate: DAF2-DA.

Diastolic Blood Pressure: DBP. L-N^G-monomethyl Arginine: L-NMMA. Mean Arterial Pressure:

MAP. Nitric Oxide: NO. Standard Erythemal Dose: SED. Ultraviolet Radiation:UV. . Systolic

Blood Pressure: SBP.

Key words: nitric oxide, ultraviolet, skin, blood pressure, photolysis, nitrate, nitrite

Abstract

The incidence of hypertension and cardiovascular disease correlates with latitude and rises in winter. The molecular basis for this remains obscure. As nitric oxide (NO) metabolites are abundant in human skin we hypothesised that exposure to UVA may mobilise NO bioactivity into the circulation to exert beneficial cardiovascular effects independently of vitamin D.

In 24 healthy volunteers irradiation of the skin with 2 Standard Erythemal Doses of UVA lowered BP, with concomitant decreases in circulating nitrate and rises in nitrite concentrations. Unexpectedly, acute dietary intervention aimed at modulating systemic nitrate availability had no effect on UV-induced hemodynamic changes, indicating that cardiovascular effects were not mediated via direct utilization of circulating nitrate. UVA irradiation of the forearm caused increased blood flow independently of NO-synthase activity, suggesting involvement of pre-formed cutaneous NO stores. Confocal fluorescence microscopy studies of human skin pre-labelled with the NO-imaging probe DAF2-DA revealed that UVA-induced NO release occurs in a NOS-independent, dose-dependent fashion, with the majority of the light-sensitive NO pool in the upper epidermis.

Collectively, our data provide mechanistic insights into an important function of the skin in modulating systemic NO bioavailability which may account for the latitudinal and seasonal variations of BP and cardiovascular disease.

Introduction.

Cardiovascular disease (CVD) accounts for 30% of deaths globally each year(Mendis *et al.*, 2011). Hypertension is a major risk factor for stroke, peripheral vascular disease, and myocardial infarction. Systolic and diastolic BP in mild hypertensives are lower in summer than winter(Brennan *et al.*, 1982), and mean population BP and hypertension prevalence increase with distance from the equator(Rostand, 1997). The same seasonal and latitude variation is seen in the incidence of acute coronary syndrome, stroke, and cerebrovascular disease(Oberg *et al.*, 2000;Zittermann *et al.*, 2005;Cottel *et al.*, 2000;Law and Morris, 1998;Rosengren *et al.*, 1999).

Incident ultraviolet radiation from the sun decreases in intensity during winter, and with increasing latitude. Ultraviolet B (UVB) wavelengths of sunlight support the synthesis of vitamin D which is essential to human health. Observational studies show an inverse relationship between serum vitamin D levels and BP and ischaemic heart disease(Lehmann *et al.*, 2001;Gouni-Berthold *et al.*, 2009); but interventional studies show no effect of vitamin D supplementation on BP, the incidence of stroke, or ischaemic heart disease(Pittas *et al.*, 2010). While vitamin D is a marker for sunlight exposure, the factors that lower BP and cardiovascular mortality thus appear to be independent of it.

We have previously identified a rich store of NO metabolites in human skin, yet its biological

role remains unknown. Nitrosothiols, nitrite and nitrate are present in significantly higher quantities in skin compared to the circulation(Mowbray *et al.*, 2009). Nitrate is the major nitrogen oxide in the dermis and epidermis where it is present at around 80 μmol/L, which is 2-3 times higher than the circulating concentration(Mowbray *et al.*, 2009;Paunel *et al.*, 2005). It is the ultimate oxidation product of NO and has long been considered biologically inert. However, dietary nitrate can lower BP following reduction to nitrite by oral bacteria(Lundberg *et al.*, 2008) and improve mitochondrial efficiency(Larsen *et al.*, 2011), suggesting possible biological activity.

We have proposed that solar UVA may mobilise nitrogen oxides from cutaneous stores to the circulation to lower BP and cardiovascular mortality(Feelisch *et al.*, 2010) and here demonstrate that UVA irradiation of the skin of normotensive human volunteers lowers BP and causes arterial vasodilatation in a NOS-independent manner. Consistent with the presence of NO storage forms in the epidermis and liberation of NO from these pools we observed a rise in circulating nitrite contemporaneously with BP reduction and a fall in plasma nitrate. These observations suggest a mechanism for the modulation of systemic NO bioactivity by the skin.

Results

Whole-Body UVA Irradiation Lowers Blood Pressure

24 resting healthy human volunteers were exposed to UVA. During and for 20 min after active irradiation the mean arterial pressure (MAP) fell significantly (3.50±0.73 mmHg, P=0.0004). Sham irradiation caused a transient fall in MAP only during irradiation (2.80±0.98 mmHg **Fig. 1A**), suggesting an additional temperature effect. Diastolic BP (DBP) fell by 4.90±0.70 mmHg during active irradiation; this was sustained for 30 min (**Fig. 1B**). These changes were greater in the active than sham irradiated group (p=0.0071). Sham irradiation caused a slight, but non-significant, reduction in DBP of 2.79±1.02 mmHg. Systolic BP (SBP) showed no change from baseline with either exposure (**Fig. 1C**). Concomitant with the drop in BP heart rate rose significantly following active (3.57±1.08 bpm) but fell slightly during and after sham irradiation (**Fig. 1D**); the difference between groups was highly significant (p=0.0012).

Effects on Blood Pressure are not Mediated by Changes in Temperature

To control for changes in temperature volunteers were covered with a metal foil 'space blanket' during irradiation which allowed the temperature rise induced by the lamp, but prevented UVA irradiation reaching the skin. Sham and active UVA irradiation groups showed a similar rise in core

temperature of less than 0.5 °C immediately after irradiation, which represented a significant change from baseline (p=0.34 between groups). Core temperature gradually returned to baseline after active irradiation, but remained slightly elevated in the sham group (**Fig. 1E**) (p=0.1022). Skin surface temperature rose by 1.53±0.05 °C and 1.76±0.06 °C in sham and active irradiation groups, respectively (p=0.7001; **Fig. 1F**), and returned similarly towards baseline after irradiation.

UVA Irradiation of Human Skin has Opposite Effects on Circulating Nitrite and Nitrate

In preliminary studies plasma nitrite levels rose following UVA irradiation. To minimize fluctuations in circulating NO metabolite levels by dietary intake variation study subjects consumed a low nitrite/nitrate diet for 2 days before further mechanistic study. Under these conditions, nitrite increased from 0.50±0.04 μM pre-UVA to 0.72±0.04 μM immediately after irradiation and remained elevated at 0.72±0.03 μM for 40 minutes post irradiation. A non-significant trend towards lower circulatory nitrite levels was observed during sham irradiation (**Fig. 2A**). These changes seemed to occur at the expense of nitrate (**Figs. 2B**). Circulatory nitrate fell significantly from 11.79±0.64 μM to 8.99±0.40 μM and remained below baseline at 9.34±0.56 μM 40 min after active irradiation only. No significant changes were observed in plasma S-nitrosothiol and vitamin D levels following active or sham irradiation (**Fig. 2C/D**); the delayed minor rise in the latter may be due to the small UVB contribution of our UVA source.

Varying Systemic Nitrate Availability does not Alter UVA-Induced Hemodynamic Effects

Although circulating nitrate levels in our subjects on the low nitrate diet were clearly reduced compared to those on a normal diet (10.7±0.6 μM vs 20-30 μM in individuals without diet control (Mowbray *et al.*, 2009)), except during active UVA irradiation, no significant differences in MAP changes were observed between the two groups (p=0.7829; **Fig. S1**). Similarly, acute elevation of circulating nitrate by a nitrate-rich drink, which resulted in levels of 107.8 ± 15.4 μM nitrate immediately before start of the irradiation protocol, affected neither kinetics nor extent of UVA-induced BP reduction from a low nitrate diet (p=0.1660 **Fig. S2**). These findings are consistent with the notion that the cardiovascular effects observed are not secondary to direct photolysis of blood constituents in the dermal vasculature but require translocation of NO bioactivity from the skin. This further suggests that systemic nitrate availability is not a limiting factor for light-induced hemodynamic changes in humans.

UVA-Induced Effects on Forearm Blood Flow are Independent of NO-Synthase Activity

Infusion of the pan-NOS inhibitor L-NMMA upstream of the vascular bed under study caused a fall from baseline of FBF within 20 min. This was sustained throughout the protocol during sham (2.34±0.21 to 1.69±0.18 mL/100mL of tissue/min, p<0.0001) and active irradiations (2.56±0.37 to

1.51±0.15 mL/100mL of tissue/min, p<0.0001; **Fig. 3A/B**) but did not affect FBF in the non-infused, contralateral arm. UVA irradiation of the L-NMMA infused arm caused local vasodilatation, with robust elevation of forearm blood flow (FBF) over the L-NMMA baseline 30 min after irradiation (23.7±6.5 % over baseline **Fig. 3A, C**). No effects on FBF were observed with sham irradiation (**Fig. 3B**), and no changes whatsoever occurred in the unirradiated contra-lateral arm (**Fig. 3D).** FBF in the L-NMMA infused forearm, significantly increased in the active compared to the sham irradiation group (p=0.0002 **Fig. 3C**), although temperature changes in both groups were almost identical (p=0.9112; **Fig. S3**). No significant change in circulating nitrite was observed after UVA irradiation of the forearm (**Figs. S4A**), probably due to the much smaller skin area exposed to light compared to the whole-body protocol. Nitrate did fall significantly from baseline 20 and 40 minutes after active irradiation of the forearm (**Fig S4B**).

UVA Exposure of Human Skin Releases NO from Storage Forms Located in the Epidermis

Ex vivo human skin pre-labelled with the NO detecting fluorochrome DAF-2DA was irradiated with the same UVA source used in our *in vivo* studies. Irradiation increased cutaneous fluorescence most markedly in the upper epidermis and stratum corneum (**Fig. 4A/B**). This was unaffected by the NOS antagonist L-NMMA (**Fig. 4C**), but completely abrogated by the NO scavenger cPTIO (**Fig. 4D;** compare to unirradiated control, **Fig. 4E**). Fluorescence intensity increased in a

fluence-dependent manner (**Fig. 4F**).

In silico ***Modelling of Nitrite Photolysis Varies with Latitude and Season***

The chemical identity of the NO-containing species, and mechanistic details of the NO translocation from skin to circulation are currently unknown. Based on our observations that NO is released from skin *in vitro* and nitrite is generated on whole-body exposure to UVA *in vivo* we surmised that nitrite, which also appears to be an intermediate in nitrate photolysis (ref. (Mowbray *et al.*, 2009), and **Fig. S5**), contributes to skin-derived NO bioactivity. The product of NO generated from nitrite photolysis *in vitro* (**Fig S6**) and incident UV energy spectrum (Sasha Madronich *et al.*, 2013) provides an estimate of the NO release from sunlight-induced nitrite photolysis at different latitudes (**Fig. 5A**) and months (**Fig. 5B**). Our data suggest that >80% of NO release is accounted for by UVA. A significant difference in calculated NO release occurs between June and December at latitudes between 40° and 60° (**Fig. 5B**).

Discussion

Current public health advice advocates avoidance of direct sunlight even in temperate climes for fear of skin cancer. Discussions about minimum levels of sunlight exposure for optimum health (focussing largely on vitamin D) are not new (Gillie, 2010;Berwick *et al.*, 2011), but arouse concerns over solar overexposure. This study demonstrates that irradiation with UVA, corresponding to natural sunlight exposure for 30 minutes at noon on a sunny day in Southern Europe, vasodilates the arterial vasculature in NOS independent fashion and reduces blood pressure independently of changes in skin temperature. The time course of plasma nitrite/nitrate changes and concomitant cardiovascular effects observed are all consistent with the photolytic release of NO from a pre-formed store and NO translocation from the skin to the circulation, but not with vitamin D production(Feelisch *et al.*, 2010). These observations support a mechanism for the modulation of systemic NO bioactivity and a possible role of the skin in cardiovascular homeostasis. As with a previous investigation(Oplander *et al.*, 2009) our study is limited inasmuch as all interventions were acute and carried out in healthy normotensive young individuals. We do not know whether UVA maintains its efficiency on repeated exposure and whether or not its BP response varies with age, gender or disease. Moreover, nothing is known yet about the exact nature of the cutaneous NO pool, its source of formation and depletion/repletion kinetics. However, if confirmed and found to also occur at lower intensity in a more chronic setting, these results will

have significant implications for public health advice and change our risk/benefit assessment for sun exposure.

We have shown that UVA exposure with two standard erythemal doses (SEDs) to one side of the body (equating to a skin surface area exposed when wearing a short-sleeved shirt and shorts) leads to a small but significant fall in mean arterial blood pressure. This compares with a maximum daily ambient UV irradiance of 45 SEDs at UK latitudes, and 60 SEDs in southern European latitudes(Diffey, 2000). Mean arterial and diastolic BP fell following irradiation, but systolic BP did not change. Diastolic BP is a function of total peripheral resistance, and its marked drop in actively irradiated subjects, despite the compensatory rise in heart rate (**Fig. 1D**) suggests that UVA vasodilates the peripheral vasculature. The relatively small BP changes we observe may appear to be of minor significance, but a 10 mmHg change in diastolic BP is responsible for a 2-fold change in cardiovascular or cerebrovascular mortality, and this risk alteration is linear(Lewington *et al.*, 2002). A reduction of diastolic BP by 5 mmHg decreases risk for stroke by 34% and coronary heart disease by 21%(MacMahon *et al.*, 1990). In fact, any amount of BP reduction is protective against stroke and cardiovascular mortality(Lewington *et al.*, 2002;Lawes *et al.*, 2004), and the magnitude of changes observed in the present study would appear to be large enough to account for the standardised mortality differences in populations living at different latitudes.

Dietary nitrate supplementation can lower BP(Lundberg *et al.*, 2008) but we found no augmentation of UVA-induced hemodynamic effects in the normal diet group compared to the low nitrate group. Opländer *et al* also demonstrated a BP reduction accompanied by increased circulatory nitrite following UVA irradiation(Oplander *et al.*, 2009), but their temperature control involved immersing subjects in warm water. This procedure will have increased the circulating volume by hydrostatic pressure on the great veins of the legs and is thus not as accurate as our sham irradiation control. In addition to BP changes we show that UVA increases FBF in a NOS and temperature-independent manner (**Fig. 3**) and a dose dependent NO release within irradiated human skin (**Fig. 4**). Our data are aligned with the well-known seasonal variation in BP at temperate latitudes, most clearly demonstrated in the MRC studies on hypertension (Brennan *et al.*, 1982). We have carefully controlled for temperature using light-impermeable aluminium foil. The sham irradiated subjects had a slightly higher rise in core and skin temperature (**Fig. 1E, 1F**), but a smaller fall in BP than the actively irradiated group. Moreover, sham irradiation produced no rise in circulating nitrite which we believe contributes to the UVA-induced fall in BP. Our FBF study showed no vasodilatation in the sham irradiated group despite the close matching of temperature with the actively irradiated subjects (**Fig. S3**).

The skin contains large stores of nitrogen oxides, particularly nitrate(Mowbray *et al.*, 2009). Our *in vivo* data showing concomitant reductions in circulating nitrate and rises in nitrite upon

UVA irradiation (**Fig. 2C**) suggest nitrate to nitrite conversion is involved in light-induced BP reduction. However, the fall in nitrate we observe is about 10 times greater than the rise in nitrite. Moreover, acute modulation of circulating nitrate by dietary means did not alter the extent of light-mediated BP changes (even after elevating plasma levels by an order of magnitude; **Figs. S1, S2**), supporting the idea that light-induced cardiovascular effects *in vivo* require bioactivation of a cutaneous rather than a circulating NO store. Notwithstanding the possibility that enzymatic nitrate to nitrite reduction(Jansson *et al.*, 2008) might be enhanced by light, the rapid onset of biochemical and hemodynamic changes strongly suggests involvement of a photolytic process. We (Rodriguez *et al.*, 2003) and others(Suschek *et al.*, 2010;Oplander *et al.*, 2010) have shown that nitrite readily yields NO on irradiation with UV. By comparison, the yield of NO from direct nitrate photolysis is very low, but thiols dramatically enhance this process (Dejam *et al.*, 2003). The SH-containing amino acid cysteine is widely present in the epidermis, either in its free form (along with other small thiols such as glutathione) or as protein constituent, in particular in the upper viable epidermal layers(Ogawa *et al.*, 1979). Cysteine-mediated enhancement of nitrate photolysis to NO, with intermediary nitrite formation, may thus be involved in the cardiovascular effects we observe (**Fig. S5**). UVA will penetrate 500 μm deep into the skin(Tuchin, 1993), so these reactions can occur in the epidermis, dermis, or even the dermal vasculature. The greatest intensity of DAF-2DA related fluorescence following UVA was in the upper epidermis and stratum

corneum, and was completely abolished by NO scavenging. These superficial sites receive the greatest UV energy, and we can speculate on the origin of these cutaneous NO stores.

Both endothelial and inducible NOS are expressed in human epidermis; generation of NO from sweat nitrate(Weller *et al.*, 1996) and conversion of ammonia to nitrite(Whitlock DR and Feelisch, 2009) occur on the skin surface. Thus, epidermal NO stores may be formed primarily from NOS activity and be replenished by "bathing" in nitrite/nitrate-containing sweat in addition to contact with extra-cellular fluid. This would not explain the drop in circulating nitrate we see upon UVA exposure; if not due to direct photolysis the latter can only mean that nitrate is taken up by cells in response to irradiation with light; this deserves further investigation. As NO rapidly diffuses over short distances, nitrite/nitrate photolysis has the potential to deliver NO to the dermal vasculature, and thus the systemic circulation. We identified pre-formed NO stores in the skin as the source of UVA induced cardiovascular effects, as these still occur following NOS inhibition (**Fig. 3**). Dilatation of forearm blood vessels, reduction in BP, and rise in circulating nitrite all lasted for at least 30 minutes after UV irradiation, indicating the involvement of longer-lived NO-containing metabolites as carriers of bioactivity. Surprisingly, plasma nitrosothiol levels did not change (**Fig. 2D**), but nitrite itself has biological activity and can nitrosylate cell/tissue constituents(Bryan *et al.*, 2005). Thus, the skin has the potential to contribute not only to cardiovascular homeostasis but also to distal organ protection by increasing the circulating NO

metabolite pool(Feelisch *et al.*, 2010).

The spectrum of incident UV at the earth's surface varies with latitude and season. Shorter wavelength radiation is preferentially filtered out by the atmosphere. As the azimuth angle decreases with increased latitude and seasonal change to winter, the thickness of the atmosphere through which solar radiation passes increases, with a consequent reduction in the UVB:UVA ratio, and overall incident energy at the earth's surface. While the exact chemical composition of the cutaneous NO pool is currently unknown, nitrite is a likely constituent. Nitrite photolysis is wavelength and energy dependent, and the product of its action spectrum with geographic and seasonal incidence data might give an indication of the rate of NO release from cutaneous stores by latitude and season (**Fig. 5**). UVB has a higher energy per photon compared to UVA, but only penetrates to the epidermis. Our calculations show that UVA is responsible for the majority of the NO release, with significant seasonal variation at latitudes between 40 and 60° (**Fig. 5B**), which correlates with the known seasonal variation in BP and cardiovascular mortality at these temperate latitudes. Less NO is produced further from the equator, which mirrors the relationship of latitude with BP and hypertension prevalence.

Serum vitamin D levels correlate inversely with BP, but vitamin D supplementation has no effect on BP and cardiovascular morbidity/mortality(Pittas *et al.*, 2010). Our data suggest that while vitamin D levels are a biomarker for sunlight exposure, the mechanism by which sunlight

lowers BP is predominantly via UVA-mediated photolysis of cutaneous nitrite and/or nitrate to NO. This finding has significant public health implications. High latitude countries such as Scotland carry a heavy burden of cardiovascular and cerebrovascular disease, and this has persisted despite interventions taken to reduce other risk factors. Concerns over rising rates of melanoma and non-melanoma skin cancers, for which excess sunlight is a risk factor, have led to advice on minimizing exposure to sunlight and maximizing the use of sunblock. Age standardised melanoma mortality(Forsea *et al.*, 2012) is much lower than that from CVD or cerebrovascular disease(Nichols *et al.*, 2012) (2.6 vs 112.5 vs 59.5 /100,000 per year in 2008). We are concerned that well-meaning advice to reduce the comparatively low numbers of deaths from skin cancer may inadvertently increase the risk of death from far higher prevalent CVD and stroke, and goes against epidemiological data showing that sunlight exposure reduces all cause and cardiovascular mortality(Yang *et al.*, 2011;Brondum-Jacobsen *et al.*, 2013)

Materials and Methods

Clinical Study Protocols

Human studies followed Declaration of Helsinki protocols and were approved by the South East Scotland Local Research Ethics Committee (LREC, reference 08/S1102/6 and 11/AL/0130) Subjects gave written, informed consent. Studies were conducted in a quiet, temperature-controlled room (22-24 °C).

Ultraviolet Light Source

A Waldmann GH-8 ST unit (Herbert Waldmann GmbH, Villingen-Schwenningen, Germany) containing 8 F851 100W UVA bulbs (320-410 nm; maximum 351 nm), producing 20 J/cm^2 at 20cm (two SEDs) was used for clinical and *ex vivo* studies.

Blood Pressure Study and Dietary Intervention

24 healthy volunteers (18 males, 6 females 22±0.5 years; Subject skin types were (1) I, (4) II, (4) III, (6) IV, (2) V and (7) not given **Tab. S1**) were randomly assigned to two groups of 12: in one, a low nitrite/nitrate diet (Wang *et al.*, 1997)was given for 2 days before UVA irradiation; in the other volunteers enjoyed an unrestricted diet. Subjects in the low nitrate group participated in a follow-up study one week later in which systemic nitrate levels were acutely raised immediately before UVA irradiation by ingestion of 500mg potassium nitrate(Larsen *et al.*, 2007).

Subjects wore underwear during irradiation. After resting supine for 30 min wearing

comfortable clothing, they were sham irradiated for 22 min. Aluminium foil blocked incident UVA radiation reaching the skin while allowing surface and body temperature rises. Following a 60 min observation period active UVA irradiation was given (20 J/cm^2 over 22 min). Subjects were monitored for a further 60 min. BP and heart rate were measured in triplicate every 10 min using a semi-automated non-invasive oscillometric sphygmomanometer. Blood was taken at 20 min intervals, centrifuged, snap frozen and stored at -80°C (Feelisch *et al.*, 2002) for later determination of plasma NO metabolites. Core temperature was measured by axillary skin (MLT422/A, AD Instruments, Chalgrove, UK) or tympanic probe (Genius^{TM} 2, Tyco Healthcare, Hampshire, UK). Surface temperature was measured by MLT422/A probes on chest and back.

Forearm Blood Flow Study

12 healthy male volunteers (22±1 years. Skin types were (4) II, (6) III, (1) IV and (1) not given) had cannulation of the non-dominant brachial artery with a 27G wire steel needle. FBF was measured in infused and non-infused arms by venous occlusion plethysmography using mercury-in-silastic strain gauges(Benjamin *et al.*, 1995). Volunteers attended on two occasions for sham (temperature control) or active UVA irradiation (20 J/cm^2) of the cannulated forearm. The sequence was randomly allocated. NOS-derived NO production was blocked by continuous intra-brachial artery infusion of L-N^G-monomethyl-arginine (L-NMMA, 8 µmoles/min) in the irradiated arm on both visits. Skin temperature was monitored and controlled with an electric fan.

Blood samples (n=11, due to difficult venous access in one subject) were drawn from irradiated and contralateral arm for NO metabolite analyses.

Measurement of Circulating NO Metabolites and Vitamin D Levels

All biochemical analyses were performed blinded, immediately after thawing samples. Nitrosothiols (RSNO) and other nitroso species were quantified using group-specific denitrosation by injection of pretreated plasma aliquots into tri-iodide-containing reaction mixture, at 60°C and constantly purged with nitrogen. NO liberated from reduction of nitrosated proteins was measured by gas phase chemiluminescence (CLD 77am sp; EcoPhysics)(Rassaf *et al.*, 2002). Oxidation products of nitric oxide (nitrite and nitrate) were quantified by a dedicated ion chromatography system (ENO20 Analyzer; Eicom), following deproteinization with methanol (1:1 v/v) and centrifugation immediately prior to loading the autosampler. Vitamin D levels were measured as total 25-hydroxy vitamin D in paired blood samples drawn before and 24h after UVA exposure using HPLC-atmospheric pressure chemical ionization-mass spectrometry(Knox *et al.*, 2009).

Fluorescence Microscopy Study

Redundant skin from abdominoplasty procedures (n=9) was snap frozen and microtomed to 5 μm sections; later incubated with 10 μM of the NO fluorochrome 4,5 -diaminofluoresceine diacetate (DAF-2DA) for one hour at room temperature(Rodriguez *et al.*, 2005). Sections were irradiated with the same light source used in human studies. Paired sections were co-incubated with 1 mM

L-NMMA or 200 μM c-PTIO. An adjacent section was conventionally stained with haemotoxylin and eosin (H&E). Sections were examined using a Leica SP5C spectral confocal laser scanning microscope and fluorescence intensities quantified using Image-Pro Plus.

Data and Statistical Analysis:

Data were analysed using SPSS (version 17.0) and Graph Pad Prism 5 and expressed as means ± standard error of the mean (SEM) unless otherwise stated. Differences were compared by one-way or two-way repeated-measures ANOVA, after Bonferroni's correction for multiple comparisons. $P<0.05$ was considered statistically significant.

Conflict of interest

None

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Figure Legends.

Figure 1. UVA lowers blood pressure independently of temperature. A) Significant reduction of mean arterial pressure during UVA irradiation was seen in both sham and active groups, but significant reduction after cessation of exposure was observed only in the active group; means ± SEM (n=24). B) Significant reduction of diastolic blood pressure from baseline was seen during and up to 30 mins after active irradiation; means ± SEM (n=24). C) No significant change in systolic BP was seen following UVA in either sham or active group. D) Heart rate rose significantly immediately after active UVA, but not sham; means ± SEM (n=24). E) Core temperature did not change significantly from baseline in active or sham irradiated groups; mean ± SEM (n=12). F) Skin temperature rose significantly from baseline in both treatment groups at all time-points, but there was no difference between groups; means ± SEM (n=12).

Figure 2. UVA increases nitrite and reduces circulating nitrate. A). Nitrite rose significantly from baseline at all time-points following active irradiation and levels were significantly higher than those in the sham irradiated group (p=0.0176); means ± SEM (n=12). B). Nitrate fell significantly from baseline at all time-points following active irradiation (p<.0001). Nitrate levels were non significantly lower in the active than sham irradiated group (p=.0642); mean ± SEM (n=12). C). Nitrosothiol (RSNO) levels did not change significantly in either group following skin

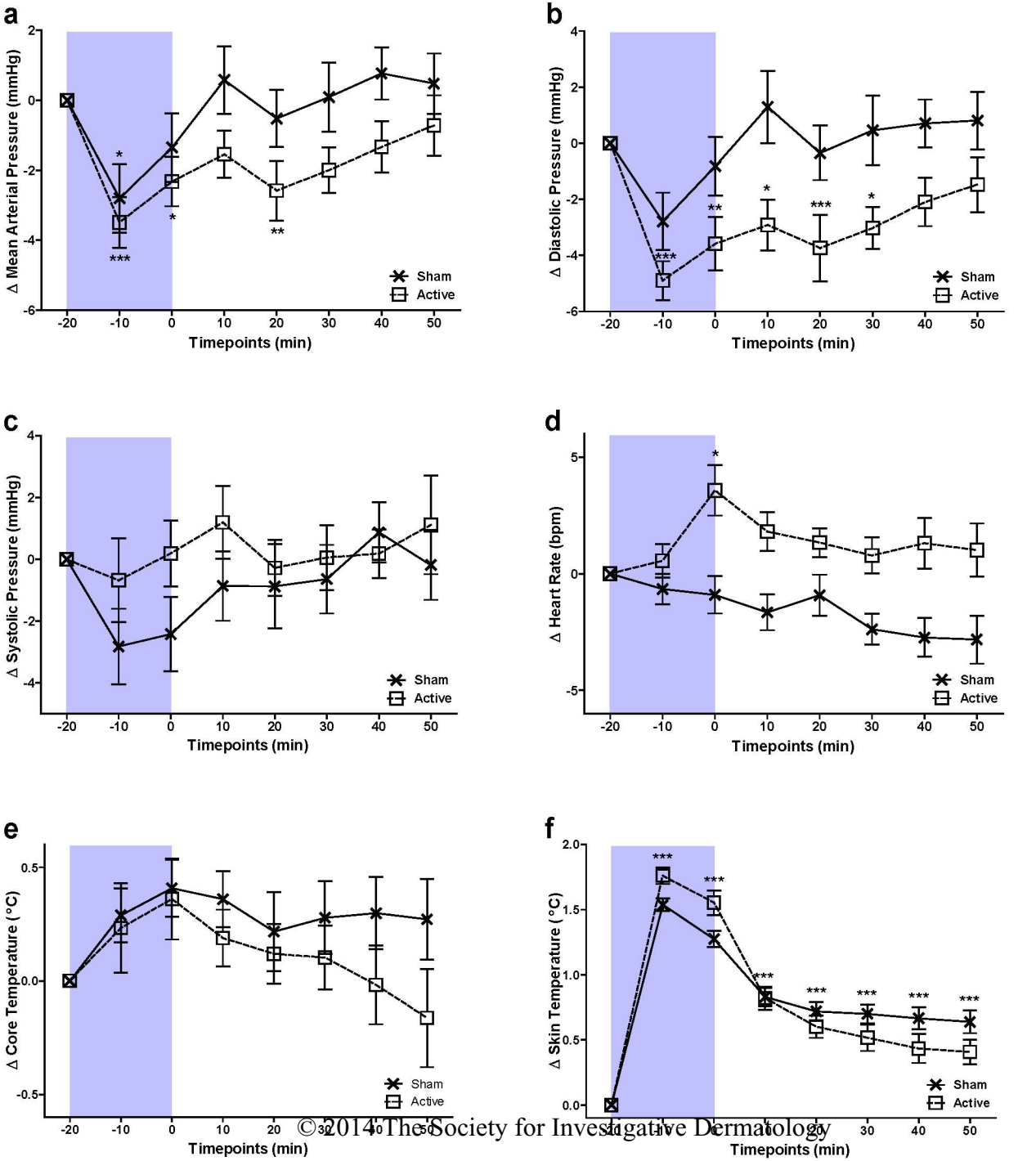
irradiation. D) Vitamin D did not alter 24 hours following active irradiation; mean ± SEM (n=12).

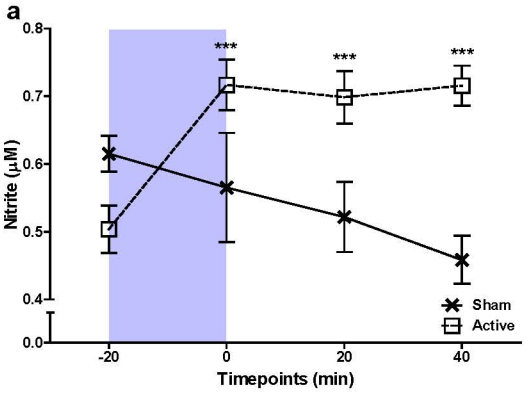
Figure 3. UVA increases forearm blood flow independently of NOS activity. A). Forearm blood flow fell significantly on L-NMMA infusion. There was no change in the non-infused, un-irradiated arm; mean ± SEM (n=12). B). Except the fall in blood flow following L-NMMA infusion, no flow change occurred in the non-infused, sham irradiated arm; mean ± SEM (n=12). C). Following active irradiation, relative forearm blood flow (FBF) rose significantly above baseline at 50 minutes. Relative FBF was significantly higher in the active than sham irradiated arm between the 20 minute (immediately post irradiation) and the 50 minute time point. p=0.0002; mean ± SEM (n=12) D). No difference in forearm blood flow was seen in the contralateral control arm in either group; mean ± SEM (n=12).

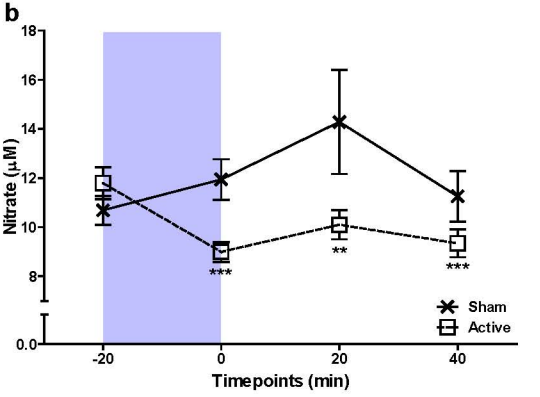
Figure 4. UVA energy-dependently liberates NO from the epidermis. A). Confocal fluorescence microscopy of human abdominal skin incubated with the NO fluorochrome DAF-2DA (10 μM) for 60 minutes, then irradiated with 45 J/cm^2 UVA. Most fluorescence is seen in the epidermis, when compared to: B) a near contiguous section of H&E stained skin. Scale bar=100μm C) Addition of L-NMMA (1 mM) had no effect on UVA-induced fluorescence. D) Addition of the NO-scavenger c-PTIO (200 μM) prevented fluorescence increase.

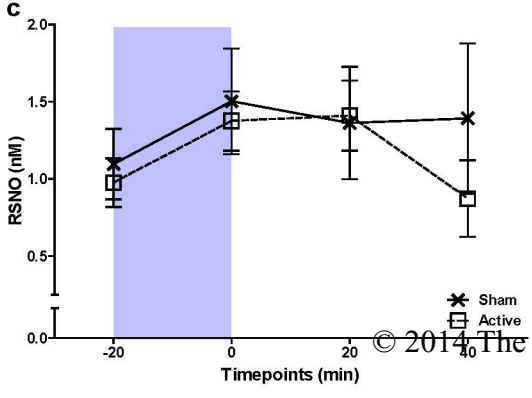
Autofluorescence of dermal fibrillar structures persists and was also seen in: E) PBS treated, unirradiated skin. F) Representative images of dose dependent fluorescence increase in actively (upper row) but not sham (lower row) irradiated skin in the range of 0 – 45 J/cm^2 UVA. Graph shows mean fluorescence ± SEM of 9 independent experiments (AU, arbitrary units). **p<.005 ****p<.0001

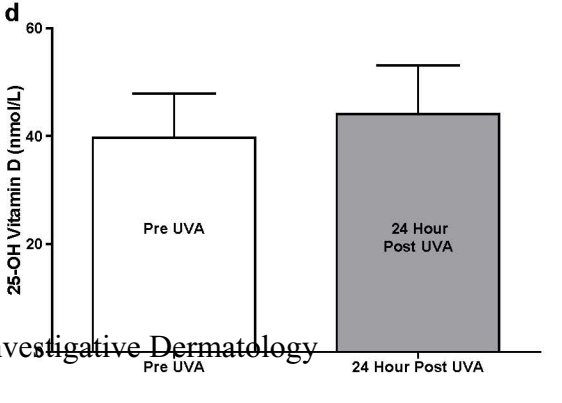
Figure 5. Calculated NO release from skin stores by season and latitude. A) Predicted NO release as the product of nitrite photolysis based on action spectrum and irradiance data by latitude. Total NO released is proportional to the area under each curve. Data are for the northern hemisphere in summer. The UVA spectrum (315~400 nm) is responsible for more than 80 % of the NO released. B) Seasonal variations in NO release between summer and winter are most marked between 30˚ and 60˚ latitude.

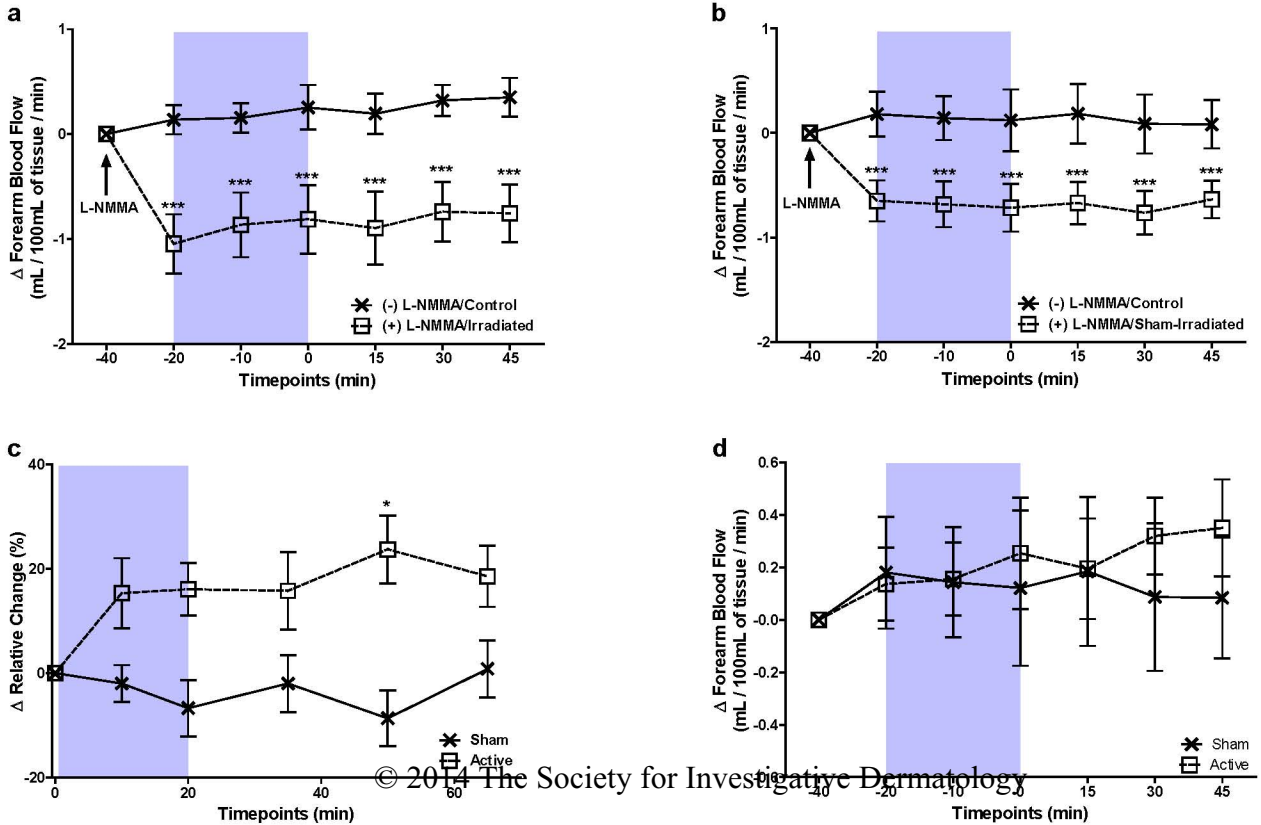


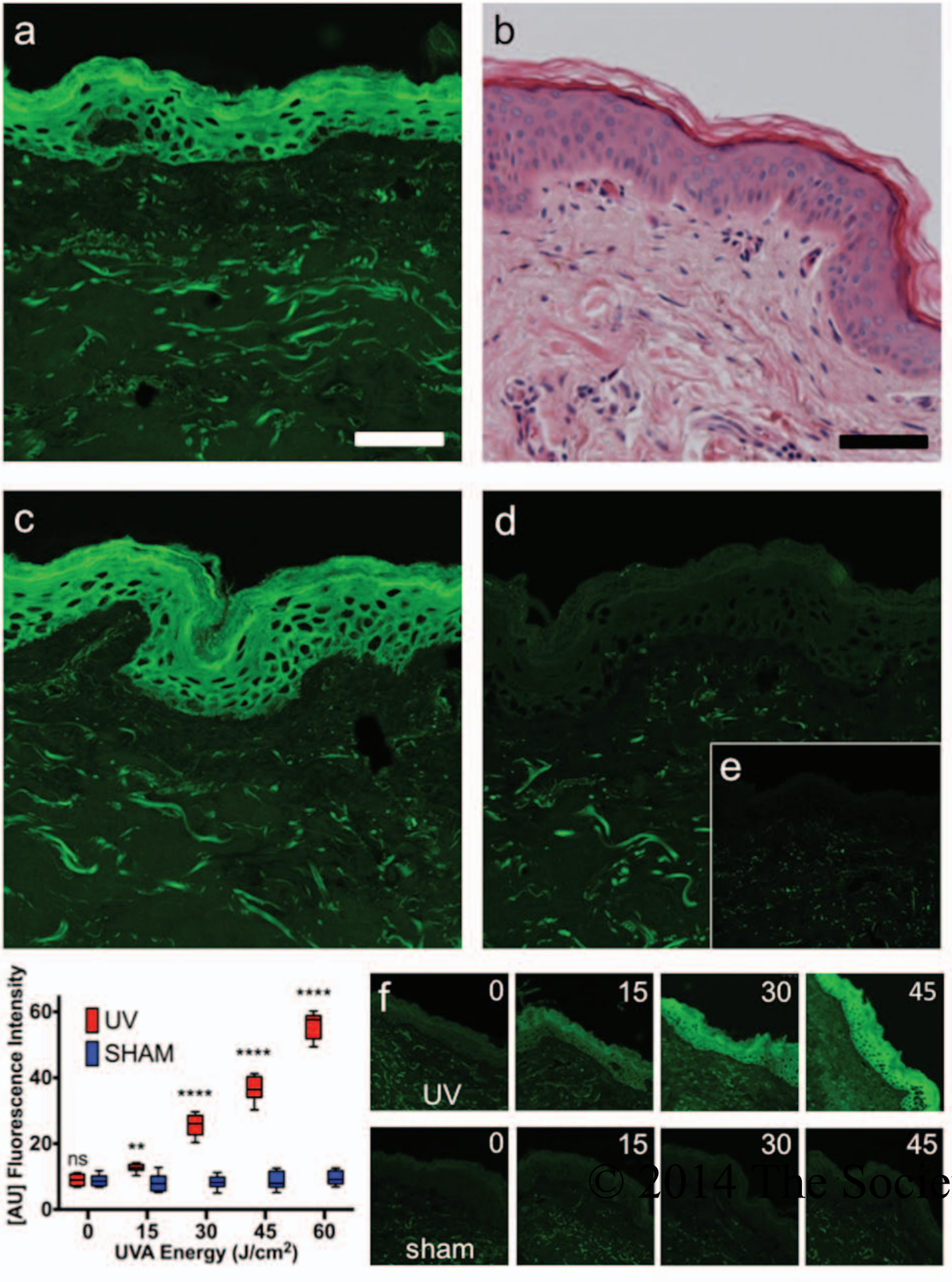


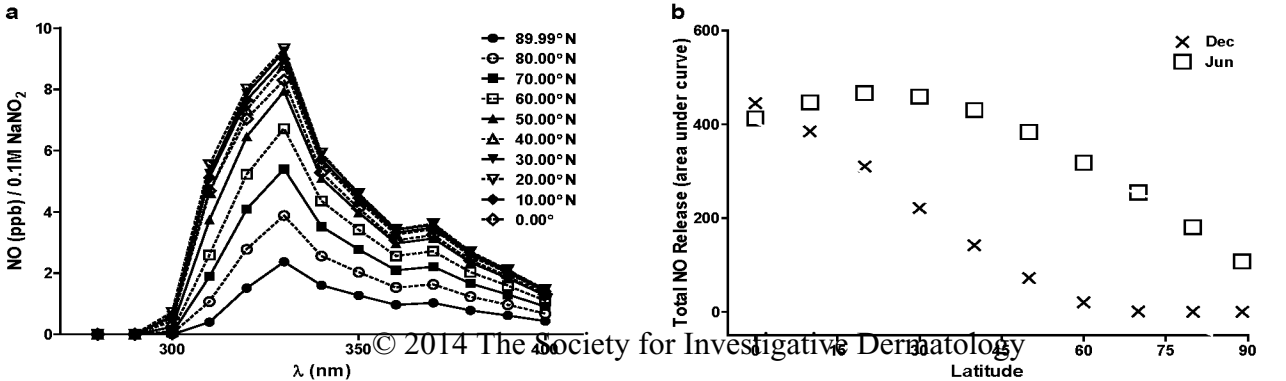












Supplementary Material

UVA Irradiation of Human Skin Vasodilates Arterial Vasculature and Lowers Blood Pressure Independently of Nitric Oxide Synthase.

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Supplementary Methods

Action spectrum of UV-induced nitrite photolysis. Aqueous sodium nitrite solutions (0.01 M) were irradiated in a reaction vessel through a quartz glass window with 1 J/cm^2 of UV light from a monochromator (Thermo Oriental, model 66921 fitted with Xe and Hg (Xe) DC arc lamp) at 10 nm intervals between 280 at 400 nm. Incubations were carried out at RT and continuous purging with nitrogen. NO release was measured with a chemiluminescence detector (42C NO-NO_2-NO_x Analyzer, Thermo Environmental Instruments) and recorded with LabChart 5 (ADInstruments).

Photolysis Studies

Data from the action spectrum for nitrite photolysis was multiplied by the solar irradiance at the

earth's surface(Sasha Madronich *et al.*, 2013) after correcting for the ozone column(Richard D.McPeters, 2013) at different latitudes and seasons. No further correction was applied for photic energy loss due to light scattering and filtering by tissue constituents.

NO release from nitrate photolysis. A separate set of experiments distinct from those on nitrite photolysis was carried out in order to investigate the effects of cysteine on UVA-induced nitrate photolysis. Reagents were placed in a reaction vessel equipped with a rubber septum-covered injection port and a quartz glass window. The reagent mixture was continuously bubbled with nitrogen through a sintered glass frit, and NO generated was detected by gas phase chemiluminescence. A bespoke cabinet containing a panel of 12 Philips TLK 40 W/10R bulbs (350–400 nm, peak 360 nm; Philips, Hamburg, Germany) delivering 10 mW/cm^2 was used as UVA source. An aqueous solution of nitrate (0.1 M) was added and irradiated with UVA in the presence and absence of cysteine (0.05 M); nitrite was scavenged by addition of acidified sulphanilamide.

Determination of the light output of the monochromator source used to construct the action spectrum of nitrite photolysis to NO. Calibration of the monochromator (Thermo Oriental, model 66921 fitted with Xe and Hg (Xe) DC arc lamp) was carried out with an Ophir 3AP optical power meter reading in mW. The lamp was warmed up and run at 1000 W (44 A, 22.8 V) and the water coolant set to 4.8C. Slit width was 1.56 mm and the UV output was

focused through a short quartz light guide to a spot of 6.6 +/- 0.1 mm in diameter. The spatial mean irradiance was calculated in mW/cm^2, and this was then converted to time to deliver $1J/cm^2$.

Supplementary tables

Supplementary Table 1. Demographics of human volunteers enrolled in the blood pressure and forearm blood flow studies.

Total	24
Gender	18♂/6♀
Age (yrs)	22 ± 0.5
Skin Type (Fitzpatrick)	I-V
Height (cm)	171.5 ± 1.9
Weight (kg)	70.4 ± 2.8
BMI (kg/m^2)	23.8 ± 0.6
Body Surface Area (m^2)	1.82 ± 0.04

Supplementary Table 2. Calibration data for monochromator used for the generation of nitrite photolysis action spectra (see Supplementary Methods for details).

Wavelength	Spatial Mean Irradiance	Time to deliver 1J/cm^2 (unweighted)
nm	mW/cm^2	sec
280	0.58	1710
290	0.88	1140
300	0.94	1069
310	1.23	814
320	1.49	671
330	1.90	526
340	2.37	422
350	2.63	380
360	2.69	372
370	2.95	339
380	3.25	308
390	3.22	311
400	3.48	287
410	3.51	285
420	3.36	297
430	3.25	308
440	3.42	292
450	3.45	290
460	3.65	274
470	4.06	246
480	3.51	285
490	3.42	292
500	3.01	332

Supplementary figures

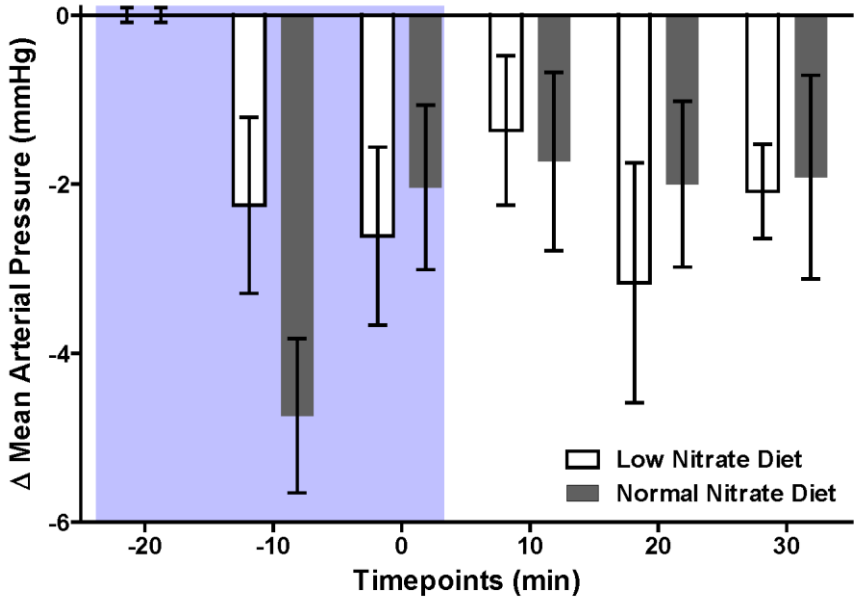

Figure S1. Mean arterial pressure changes during and after active UVA irradiation in the normal and low nitrate group. No significant difference in mean arterial blood pressure changes following UVA was observed between the normal and the low nitrate study arms. Mean ± SEM (n=12)

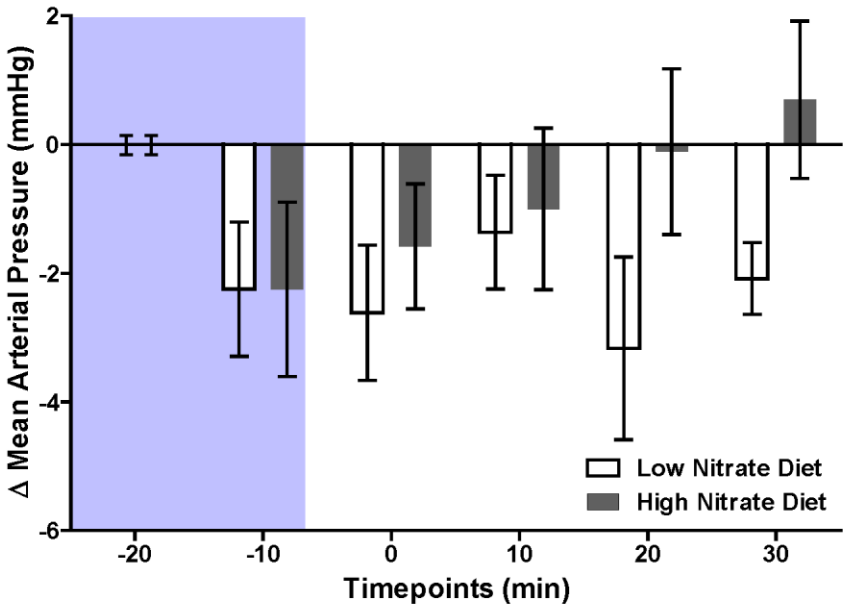

Figure S2. Mean arterial pressure change s during and after active UVA irradiation in the high and low nitrate group. No significant difference in mean arterial blood pressure changes following UVA was observed between the high and the low nitrate study arms.

Mean ± SEM (n=12)

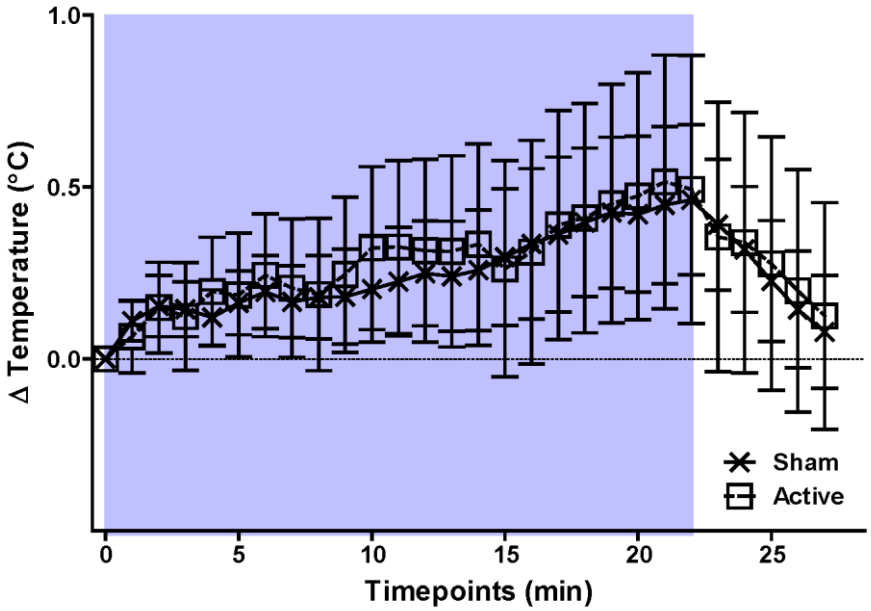

Figure S3. Time course of changes in skin temperature in active and sham irradiated forearms. Forearm skin temperature showed a non-significant upward trend during both sham and active UVA irradiation, but there was no difference between treatment arms during and after the exposure. Mean ± SEM (n=12)

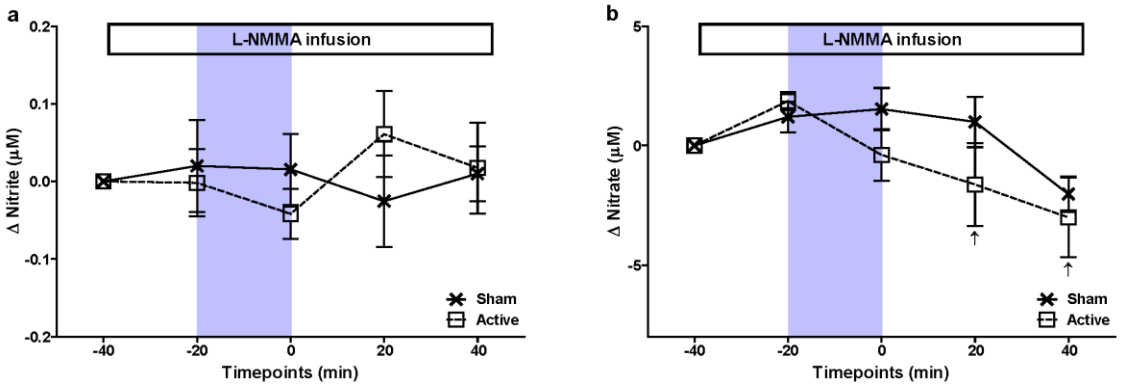

Figure S4. Changes in circulating nitrite and nitrate concentrations in the human forearm upon UVA irradiation in the presence of the NO synthase inhibitor L-NMMA. A) Plasma nitrite did not alter from baseline in the L-NMMA infused forearm in either active or sham irradiated group. B) Plasma nitrate fell from baseline 20 and 40 minutes after irradiation, but was not significantly different from the sham irradiated, L-NMMA infused forearm. The non-significant trend to a fall in forearm nitrate in the sham irradiated arm may represent the start of a systemic inhibition of NOS-dependent NO production due to the continuous L-NMMA infusion. Mean ± SEM (n=11) ↑ =p<.05

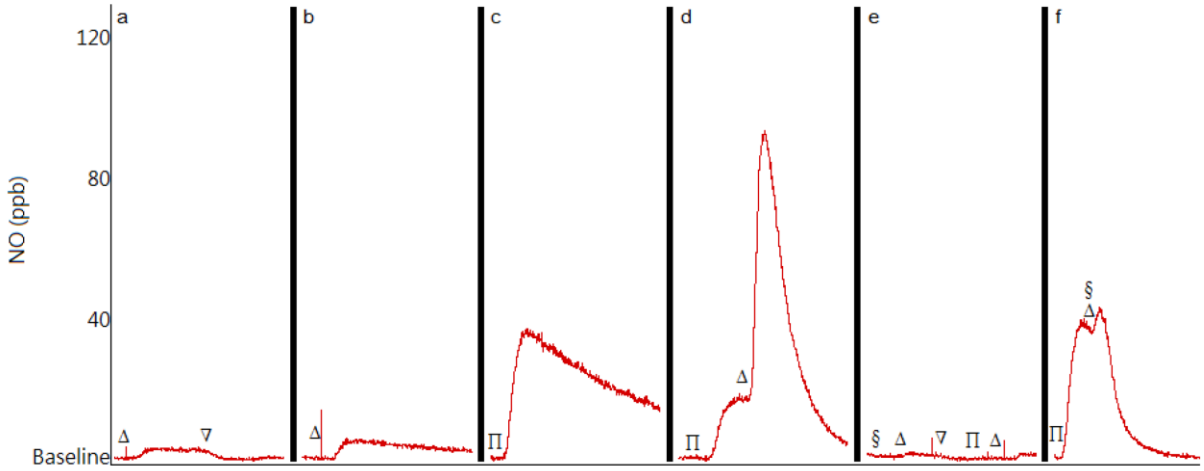

Figure S5. Cysteine potentiates UVA-induced NO release from nitrate photolysis. UVA irradiation of aqueous cysteine hydrochloride (a) or potassium nitrate solution (b) alone produced minimal NO release, which is due to trace contamination of commercial salts and buffers with nitrite. Addition of 0.1 M KNO_3 to 0.05 M cysteine solution (c) induced NO release and this was markedly augmented by UVA irradiation (d). Addition of acidified sulfanilamide prevented NO release following nitrate addition (e) and the augmented release of NO following UVA irradiation of cysteine and nitrate containing solutions(f). △ - UVA lamp switched on, ▽ - UVA lamp switched off; ∏ - addition of nitrate solution, § - addition of sulphanilamide. Depicted tracings are representative of 3-4 independent experiments.

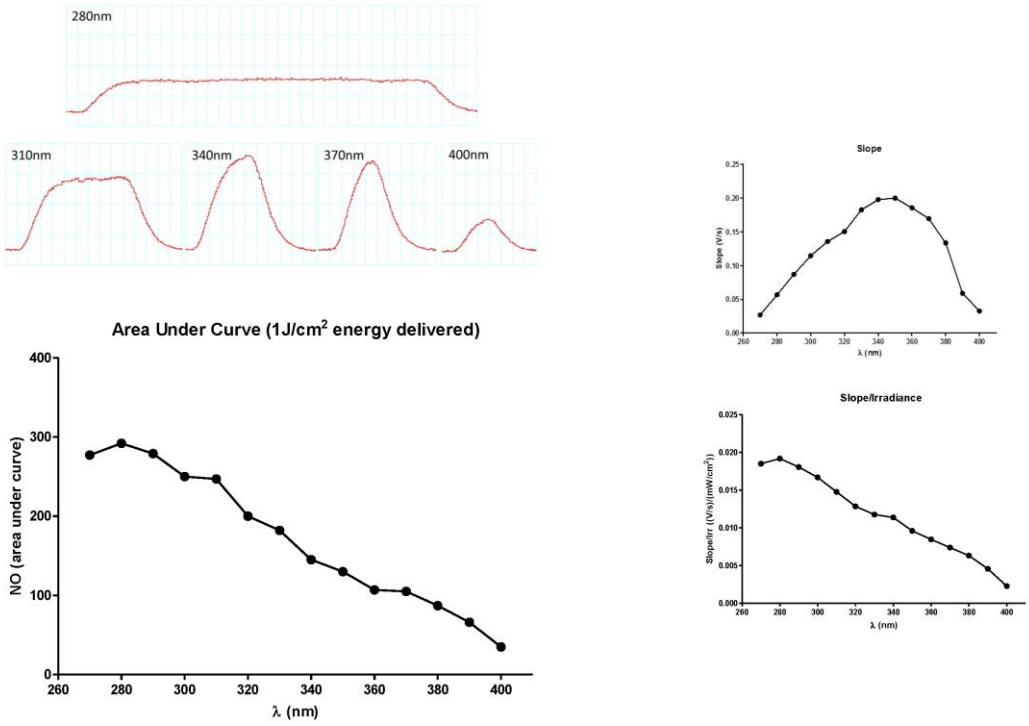

Figure S6. Action spectrum of UV-induced nitrite photolysis to NO. *Left-hand panels*: Original tracings of NO release generated during irradiation of aqueous sodium nitrite solutions with a fixed energy of 1 J/cm^2 at representative wavelengths between 280 and 400 nm. λ-dependent energy output of the monochromator used in these studies was determined with the aid of an optical power meter (see Suppl. Tab 2 for details), as described in the *Supplementary Methods* above, with differences in photic energy levels translating into different durations of exposure for a given wavelength to arrive at a matching total irradiation level of 1 J/cm^2. The area under the curve of the NO concentration vs time profile was used to calculate the total amounts of NO released upon nitrite photolysis for a given wavelength as depicted below. *Right-hand panels*: Identical results

were obtained when the slope of the NO concentration increase was plotted against the irradiation wavelength (top right) and corrected for differences in irradiance at each λ (bottom right).

Reference List

1 **Richard D.McPeters: Ozone Over Your House (NASA, 2013).**

2 **Sasha Madronich, Xu Xie Tie, Mary Barth, Danny McKenna: QUICK TUV CALCULATOR (National Center for Atmospheric Research, Boulder, CO 80301 2013).**