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Involvement of Oxygen Free Radicals in Ischaemia-Reperfusion Injury to Murine Tumours: Role of Nitric Oxide

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Ischaemia-reperfusion (I/R) injury is a model system of oxidative stress and a potential anti-cancer therapy. Tumour cytotoxicity follows oxygen radical damage to the vasculature which is modulated by tumour production of the vasoactive agent, nitric oxide ($NO^{\bullet}$). *In vivo* hydroxylation of salicylate, to 2,3- and 2,5-dihydroxybenzoate (DHBs), was used to measure the generation of hydroxyl radicals ($OH^{\bullet}$) following temporary vascular occlusion in two murine tumours (with widely differing capacity to produce $NO^{\bullet}$) and normal skin. Significantly greater $OH^{\bullet}$ generation followed I/R of murine adenocarcinoma CaNT tumours (low $NO^{\bullet}$ production) compared to round cell sarcoma SaS tumours (high $NO^{\bullet}$ production) and normal skin. These data suggest that tumour production of $NO^{\bullet}$ confers resistance to I/R injury, in part by reducing production of oxygen radicals and oxidative stress to the vasculature. Inhibition of NO synthase (NOS), during vascular reperfusion, significantly increased $OH^{\bullet}$ generation in both tumour types, but not skin. This increase in cytotoxicity suggests oxidative injury may be attenuation by tumour production of $NO^{\bullet}$. Hydroxyl radical generation following I/R injury correlated with vascular damage and response of tumours *in vivo*, but not skin, which indicates a potential therapeutic benefit from this approach.

Keywords: Tumour, vasculature, ischaemia-reperfusion injury, lactate dehydrogenase, hydroxyl radicals, nitric oxide, oxidative stress

INTRODUCTION

The reliance of many tumour cells on the function of single vascular capillaries and the abnormal structure of the tumour vasculature make it a potential target for cancer therapy. Recent studies have shown that ischaemia-reperfusion (I/R) injury, reperfusion following temporary cessation of tumour blood flow, causes significant cytotoxicity and regression of solid experimental tumours.[1–3] Studies of murine and human tumours using laser-Doppler flowmetry have shown spontaneous changes in erythrocyte flux of between 2- and 5-fold.[4,5] These changes are evidence of vascular instability which may themselves result in cycles of modest I/R injury and

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warrant further investigation of the determinants of solid tumour response to I/R injury. The cytotoxic action of I/R injury has been widely studied in normal tissues and is mediated by oxidative stress following production, initially, of superoxide radical ($O_2^{\bullet -}$).[6,7] An additional role is played by activation of neutrophils, releasing $O_2^{\bullet -}$ and hypochlorous acid, with adhesion of neutrophils to vascular endothelium.[8] Iron-dependent generation of the highly reactive $OH^{\bullet}$, by Haber–Weiss reaction between $O_2^{\bullet -}$ and hydrogen peroxide, results in damage to cell membranes by lipid peroxidation and increased vascular permeability.[3,9–14] Oxidative damage is also identified in other disease states (e.g. neurodegenerative diseases, arthritis, stroke) and some cancer therapies exert part of their cytotoxic action by production of oxygen radicals (e.g. some chemotherapy agents, photodynamic therapy (light-dependent production of $O_2^{\bullet -}$) and biological response modifiers (production of $O_2^{\bullet -}$ by tumour necrosis factor-α and interleukin-1) although the relationship to cytotoxicity is poorly understood.[15–18] Tumours show elevated hydrogen peroxide production and modified levels of anti-oxidant enzymes which indicate a condition of persistent oxidative stress and may differentiate their response to oxidative stress from that of normal tissues.[19–25] Increased tumour cytotoxicity may therefore be expected if either production of oxygen radicals were increased or the cellular anti-oxidant defenses compromised.[26–28] Carbogen ($95\%O_2 : 5\%CO_2$) breathing was used in the present study as it has been reported to improve cancer radiotherapy,[29] which itself is mediated by production of oxygen radicals.[30]

Using physical occlusion of blood supply to subcutaneous murine tumours, we have previously reported significant tumour cytotoxicity if reperfusion was allowed to follow a period of temporary vascular occlusion.[3] I/R resulted in prolonged reduction of tumour blood flow in the murine CaNT tumour ($32 \pm 5\%$ of control), contrasting with an increased (hyperemic) response measured in skin ($143 \pm 11\%$ of control) following 1 h occlusion and 1 h reperfusion.[31] Vascular occlusion also resulted in significant accumulation of tumour lactate which was rapidly restored towards control levels following clamp removal; indirectly indicating vascular reperfusion.[14] That oxygen radicals were involved during tumour I/R was inferred by the observed reduction of cytotoxicity if superoxide dismutase (SOD) or catalase (CAT) were infused into the vasculature at the time of initial reperfusion.[3] Co-administration of CAT or desferrioxamine (DFO) were used in the present study to study the role of hydrogen peroxide and iron-dependent release of $OH^{\bullet}$ from reaction between hydrogen peroxide and $O_2^{\bullet -}$.[32] The hypoxia-dependent conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) catalyses the conversion of oxygen to $O_2^{\bullet -}$ following I/R injury. The ratio of XO to XDH, which was higher in control CaNT (~38%) than SaS (~12%) tumours,[33] was significantly increased by vascular occlusion (5-fold increase after 12 h occlusion), although total enzyme activity was gradually degraded (~50%) over the same period. These changes are likely to contribute to any observed tumour-dependent sensitivity to I/R injury.

Nitric oxide ($NO^{\bullet}$) is a key molecule in control of vascular tone and also determines the adhesion of leukocytes to endothelium following I/R injury.[34–36] However, conflicting reports of both protective and deleterious effects of $NO^{\bullet}$ in clinical studies suggest it is not the sole determinant of I/R injury, potentially due to interaction between $NO^{\bullet}$ and $O_2^{\bullet -}$.[37–40] We reported that $NO^{\bullet}$ production by experimental murine and human tumours conferred protection against oxidative stress following I/R injury and photodynamic therapy.[3,41] Tumour cytotoxicity was increased by inhibition of NOS or decreased by chemicals that released $NO^{\bullet}$ at the time of reperfusion of the tumour, suggesting $NO^{\bullet}$ protects against oxidative stress. Potentiation of cytotoxicity following inhibition of NOS may be

due to: (i) increased adhesion of leukocytes, especially neutrophils, and their associated oxidative burst when they become activated; or (ii) prolonged half-life of $O_2^{\bullet -}$ by decreased formation of peroxynitrite.[36,42] Activated neutrophils produce large amounts of cytotoxic hypochlorous acid, via myeloperoxidase, and recent studies have shown that this may act as a better substrate than hydrogen peroxide for production of $OH^{\bullet}$.[13]

Administration of salicylate acts as an efficient $OH^{\bullet}$ trap yielding 2,3- and 2,5-DHBs in tissue studies of ischemia-reperfusion injury of intestine and heart tissue.[43–45] The production of 2,3-DHB, rather than 2,5-DHB, has been reported to be a better indicator of $OH^{\bullet}$ attack on salicylate[46] although the production of the latter product may be artefactually increased by metal parts in microdialysis equipment.[47] In addition it has recently been reported that DHBs can be formed by $OH^{\bullet -}$ released when peroxynitrite (formed by combination of $O_2^{\bullet -}$ and $NO^{\bullet}$) or other $ONOO^-$-derived species break down.[48,49] Interpretation of the assay should therefore be made with caution. Inhibition of $OH^{\bullet}$ generation has been shown to be modulated by inhibition of NOS activity in brain tissue.[50]

It was the purpose of the present experiments to determine the role of oxygen radicals and $NO^{\bullet}$ in the cytotoxic action in a murine tumour model of I/R injury. Any potential therapeutic benefit of this type of treatment was assessed by comparison of the response of tumours with normal skin.

MATERIALS AND METHODS

Animals and Ischaemia-Reperfusion Model

Female CBA/Gy f T0 mice, aged 12–16 weeks, were used throughout this study. Mice were implanted, subcutaneously, with $\sim 1 \times 10^6$ cells of the syngeneic breast adenocarcinoma CaNT or sarcoma SaS. Tumours were grown until their geometric mean diameter was approximately 8 mm (~300 mg) before treatment. Tumour vascular supply was temporarily clamped using a hinged metal 'D'-clamp which had been placed over the tumour, thereby clamping the supplying vessels and skin.[51] The clamps were held closed by spring clips for the duration of occlusion and were removed to allow vascular reperfusion. Immediately prior to clamp removal, sodium salicylate (2-hydroxy benzoic acid) (Merck Ltd. GPR grade) was administered via intraperitoneal (ip) injection (50 mg/kg). Salicylate is therefore carried through the tissue during vascular reperfusion and reacts with hydroxyl radicals to form DHBs. N^{ω}-nitro-L-arginine (L-NNA) (20 mg/kg), catalase (3000 U/ml) or desferrioxamine (100 mg/kg) were dissolved in saline. Each agent was administered ip at the same time as salicylate. Immediately after clamp removal, mice were placed inside a large perspex box of 20 l capacity and gassed continuously (5 l/min) with either air (21% oxygen) or carbogen (95% oxygen/5% carbon dioxide) throughout the reperfusion period. After 1 h of reperfusion, mice were killed by cervical dislocation and blood collected in heparinised syringes (Unihep Leo 5000 IU/ml, Leo Laboratories, UK) from the opened thorax. Plasma was separated from whole blood by centrifugation (4500 rpm for 5 min). Plasma samples were stored frozen at −20°C until analysis. Normal skin on the dorsum was also studied in a similar manner to tumours. All experiments were performed in accordance with the UK Home Office regulations in the Animals (Scientific Procedures) Act 1986.

Lactate Dehydrogenase Activity

The release of lactate dehydrogenase from endothelial cells is reported following oxidative injury.[52–54] Subcutaneous CaNT or SaS tumours or normal skin were clamped as described in the previous section. At various times after clamp removal, animals were killed and whole blood collected in heparinised syringes. Plasma was obtained after separation by centrifugation and

aliquots stored at −20°C until analysis. Plasma lactate dehydrogenase activity (IU/ml) was measured by an automated colorimetric assay measuring the conversion of pyruvate to lactate.

HPLC Analysis

Plasma samples were assayed for the concentration of DHBs and parent salicylate by HPLC (Waters) following extraction with 10% trichloracetic acid. A gradient separation method was used as follows: column Hichrom RPB (100 × 4.6 mm) (Hichrom, Reading); eluents A: 15 mM KH_2PO_4, 15 mM H_3PO_4; B: methanol; linear gradient 31–50% B over 2–6 min; flow rate 2 ml/min. The concentration of salicylate was determined using absorbance detection at 290 nm and electrochemical detection was used for 2,3- and 2,5-DHBs. Calibration was performed using known concentrations of stock solutions.

Results are presented as the percentage of plasma salicylate converted to the 2,3- or 2,5-DHBs relative to total salicylate measured in the same sample. Pharmacokinetic studies indicated that the concentration of both DHBs were relatively stable after 1 h reperfusion.

RESULTS

Hydroxylation of Salicylate following Ischaemia-Reperfusion

Pharmacokinetic studies of the plasma levels of sodium salicylate and its hydroxylated products, 2,3- and 2,5-DHBs were made in tumour bearing mice. Salicylate reached peak levels of >0.5 mM for at least 1 h following ip injection at 50 mg/kg and during 1 h circulation a proportion of salicylate is converted to 2,5-DHB (4.68% ± 0.52) and significantly less to 2,3-DHB (0.12% ± 0.02) in control mice (data expressed as the fraction of DHB as a percentage of the plasma salicylate concentration). Plasma levels of the DHBs appear relatively stable during this time and it was decided to measure the effect of I/R and other modulators at 1 h following salicylate injection and clamp removal. Figure 1 shows the production of DHBs measured in plasma following 1 h clamp of skin or CaNT tumour followed by 1 h of reperfusion whilst breathing air or carbogen. The production of DHBs by non-tumour bearing mice, in air or carbogen for 1 h, is shown as control. The production of 2,3-DHB following I/R injury was significantly elevated in both tissues by breathing carbogen, compared to air, during the reperfusion period ($p < 0.005$ for both tissues). Only the production of 2,3-DHB following CaNT tumour reperfusion in carbogen was significantly higher than control ($p < 0.05$). The tumour production of 2,5-DHB was significantly

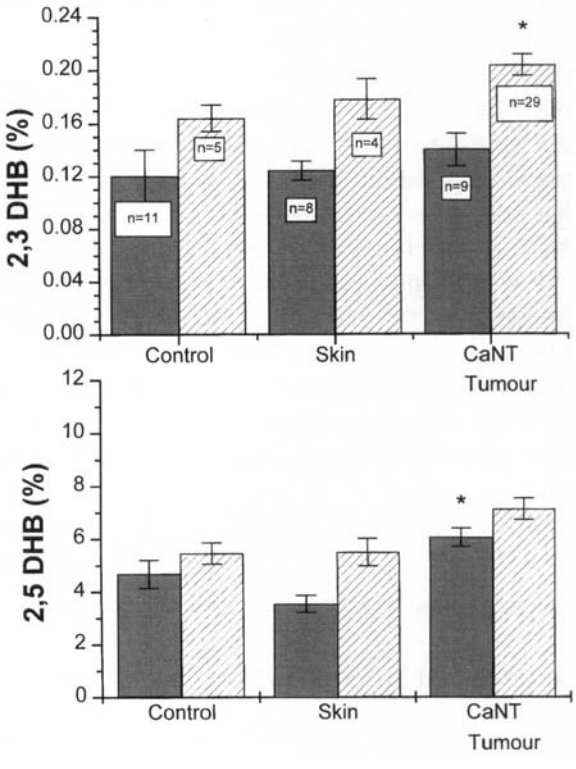

FIGURE 1 Yield of plasma 2,3- and 2,5-DHB, hydroxylation products of salicylate, following I/R injury to normal skin or CaNT tumour (1 h occlusion and 1 h reperfusion) whilst breathing air (filled bars) or 95% oxygen (hatched bars). (* denotes $p < 0.05$ compared to control) (n = number of samples).

elevated compared to control whilst breathing air ($p < 0.05$) although statistical significance was not reached for carbogen breathing ($p = 0.09$). Production of 2,5-DHB by skin was not significantly elevated by carbogen breathing during reperfusion. Overall, the production of DHBs by reperfused CaNT tumours was increased to a greater degree above control than that observed following skin reperfusion.

Effect of Inhibition of NOS on Hydroxylation of Salicylate

Figure 2 shows the effect of inhibition of NOS on $OH^{\bullet}$ production in skin compared to the CaNT and SaS murine tumours, whilst breathing carbogen. The production of DHBs by non-tumour bearing mice, breathing carbogen, are shown as control. Inhibition of NOS during reperfusion resulted in significantly higher levels of DHBs in the CaNT tumour but not in the normal skin or SaS tumour. The SaS tumour shows significantly lower production of DHBs than the CaNT tumour, as may be expected by its relative insensitivity to clamp-reperfusion *in vivo*. It is clear that inhibition of NOS during reperfusion of the CaNT tumour resulted in significant elevation of both 2,3-DHB ($p < 0.05$) and 2,5-DHB ($p < 0.05$). Inhibition of NOS in the SaS tumour produced a less pronounced increase of DHBs with only 2,5-DHB reaching statistical significance. Inhibition of NOS during skin reperfusion did not result in significant elevation of either DHBs.

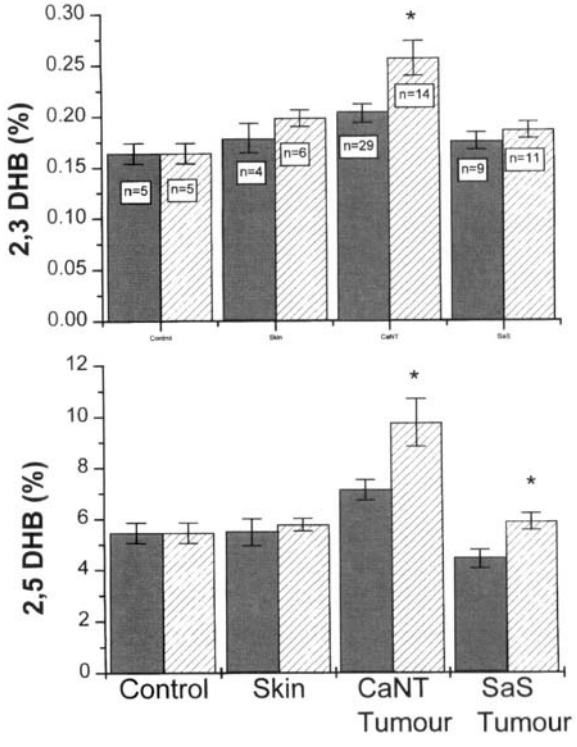

FIGURE 2 Yield of plasma 2,3- and 2,5-DHB formed following I/R injury to skin, CaNT and SaS tumours whilst breathing 95% oxygen (filled bars) or following inhibition of NOS (hatched bars), by iv injection of N^{ω}-nitro-L-arginine [20 mg/kg] at the time of clamp removal (1 h occlusion, 1 h reprefusion) (*denotes $p < 0.05$ for effect of NOS inhibition) (n = number of samples).

In this study there was significant correlation between the plasma production of 2,3-DHB and 2,5-DHBs following I/R injury in tumours (overall correlation coefficient $R = 0.77$, $P = 1.82\text{E}{-10}$, $n = 48$) (Figure 3). The degree of tumour cytotoxicity following I/R injury has been shown to be related to duration of ischaemia and subsequent reduction in vascular perfusion, although the relationship to production of $OH^{\bullet}$ was previously unmeasured.[3] The generation of both DHBs were enhanced by inhibition of NOS, in agreement with previous measurements of increased tumour cytotoxicity.

Effect of Infusion of Catalase or Desferrioxamine

Catalase (CAT) or desferrioxamine (DFO) were given by intravenous injection just prior to clamp removal, i.e. at the time of reperfusion, and resulted in significantly reduced production of both 2,3- and 2,5-DHBs in the CaNT tumour (Figure 4). These data indicate that the iron-dependent reaction between $O_2^{\bullet -}$ and hydrogen peroxide, as evidenced by catalase-dependent reduction of hydrogen peroxide or iron-chelation

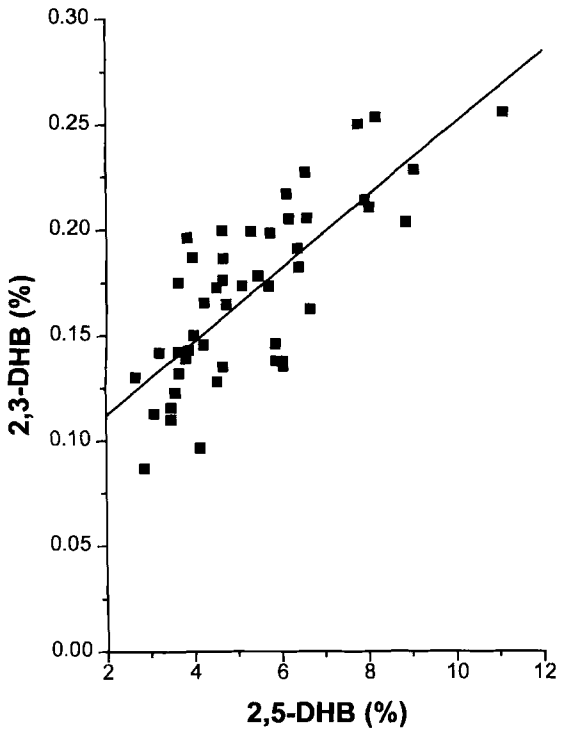

FIGURE 3 Correlation between plasma level of 2,3- and 2,5-DHB formed following salicylate administration to control or clamp-reperfused skin or tumours ($R = 0.77 \pm 0.03$ sd, $p = 1.82E{-}10$, $N = 48$).

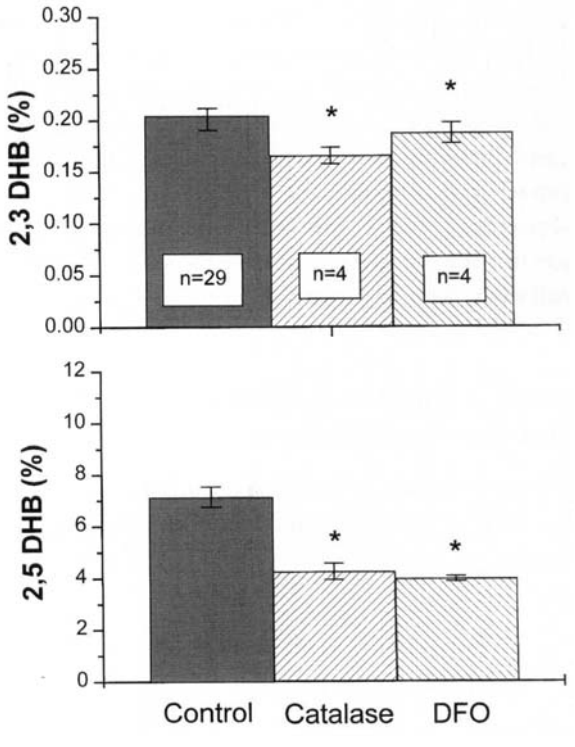

FIGURE 4 Yield of 2,3- and 2,5-DHB when catalase (CAT) or desferrioxamine (DFO) was administered into systemic vasculature at time of clamp removal and 1 h reperfusion following 1 h vascular occlusion (carbogen breathing) (* denotes $p < 0.05$) (n = number of samples).

TABLE I Plasma LDH Activity (IU/ml)

Tissue	Control	1 h clamp + 15 min reperfusion	1 h clamp + 1 h reperfusion
No tumour/skin	690 ± 140	N/A	1070 ± 300
CaNT tumour	1220 ± 430	3350 ± 440	2280 ± 580
SaS tumour	1160 ± 30	780 ± 100	N/A

N/A = not measured.

by DFO, are important in determining $OH^{\bullet}$ generation.

Effect of I/R Injury on Lactate Dehydrogenase in Endothelial Cells

Measurement of plasma lactate dehydrogenase (LDH) was made in control mice and at 0.25 or 1 h following release of a 1 h clamp of either CaNT, SaS tumours or normal skin (Table I). The results indicate that the presence of untreated murine tumours elevates LDH activity above that of non-tumour bearing mice. Following reperfusion, plasma LDH activity is significantly elevated (>2-fold) by the CaNT tumour within 15 min after clamp release. The plasma LDH activity following a similar treatment to SaS tumour was not significantly different from untreated, non-tumour bearing mice. The plasma LDH activity at 1 h shows a long-lasting elevation by CaNT

tumour reperfusion with no significant elevation by skin reperfusion.

DISCUSSION

It was the aim of this study to determine the generation of hydroxyl radicals following I/R injury to murine tumours and skin. The data show that the highest concentration of plasma-hydroxylated products (DHBs) were produced following reperfusion of the CaNT tumour which shows the greater *in vivo* response compared to the SaS tumour or normal skin. Evidence of vascular damage is clear from elevated plasma concentration of LDH following I/R injury in CaNT tumours and is consistent with greater endothelial cell cytotoxicity than for a similar treatment to SaS tumour or normal skin. Taken together the data suggest vascular damage to the CaNT tumour is mediated, in part, by generation of $OH^{\bullet}$ radicals. The data presented in this report indicate the generation of $OH^{\bullet}$ during the first hour of reperfusion following clamp removal. As such they cannot directly indicate the total exposure of the tissue to $OH^{\bullet}$ which may occur for longer duration following initial reperfusion. Under control conditions it is reported that tumours show indications of persistent oxidative stress, possibly due to reduced anti-oxidant protection.[25] It is therefore likely that cancer therapy by I/R injury could be improved if the generation of oxygen radicals, and greater oxidative stress, were enhanced.[27,55] The modification of vascular damage, following intravenous injection of SOD, CAT and DFO indicates that oxidative injury can be modified. Elevated inspired oxygen concentration was used in this study as it was expected to amplify the generation of oxygen radicals and allow a clearer distinction between the different tissues and their response to inhibition of NOS.

We have previously shown that tumour production of $NO^{\bullet}$ plays an important role in protecting tumour vasculature from I/R injury with significant heterogeneity between tumour types.[3] $NO^{\bullet}$ production also correlates with the histological grade of human breast and gynecological tumours.[56,57] It is likely therefore that $NO^{\bullet}$ may determine tumour cytotoxicity to other cancer therapies exerting their action by oxidative stress. However $NO^{\bullet}$ may not be the sole determinant of tissue response to I/R injury as was evidenced from the hyperemic response of skin which has very low $NO^{\bullet}$ production. It is probable that the abnormal structure and function of vasculature within solid tumours plays an enhancing role in the observed sensitivity of tumour vasculature reported in this paper.

Salicylate reacts with $OH^{\bullet}$ forming DHBs as previously described. However it has been commented that production of 2,5-DHB may also be increased by cytochrome P-450 and therefore not solely due to $OH^{\bullet}$ attack although production of 2,3-DHB by P-450 is less likely and is therefore a better indicator of $OH^{\bullet}$ generation.[47,58] In the present study the concentration of both DHBs increased following clamp removal and was clearly dependent upon the concentration of inspired oxygen. This suggests that elevated blood oxygenation increases the production of $OH^{\bullet}$ from reperfused tissue. The relative generation of both DHBs was in general agreement with that reported for other tissues, e.g. reperfused small intestine.[46] However the significant increase in systemic plasma concentration of DHBs, especially following NOS inhibition, is likely to underestimate the local oxidative stress occurring within the tumour microcirculation.

The difference in sensitivity to I/R injury shown by the two murine tumours in this report appears intimately linked to their intrinsic capacity to produce $NO^{\bullet}$. Nitric oxide produced via the constitutive NOS in endothelial cells (ecNOS) has a vasorelaxant effect on smooth muscle as well as an anti-adhesive action on host leukocyte/endothelium binding. The induced form of NOS (iNOS) is present in tumour cells and activated macrophages. Precisely whether it

is the activity of the tumour iNOS or ecNOS, or a combination of both, that confers resistance to I/R in the SaS tumour is part of our ongoing studies. However it has been shown that tumour NOS activity is correlated with response to photodynamic therapy, a cancer therapy mediated by production of $O_2^{\bullet -}$.[41] Inhibition of NOS in this study resulted in greater production of $OH^{\bullet}$ by the tumour type with modest NOS activity (CaNT) suggesting the balance between the protective role of $NO^{\bullet}$ and oxidative stress by oxygen radicals could be easily compromised. The higher NOS activity of the SaS tumour conferred greater protection against I/R injury with modest enhancement of damage when NOS was inhibited. These observations are supported by observations of the protective action of NO in some biological systems.[42]

Nitric oxide can react directly with oxygen radicals to form peroxynitrite, which is a potent oxidant and may mediate cellular injury by release of hydroxyl-like radicals.[48–50,59] However the present results for SaS tumours, which have high NOS activity, suggest that peroxynitrite, if formed, is not associated with increased hydroxylation of salicylate. The conflicting expectation of increased peroxynitrite, formed because of its relatively elevated NOS activity, but observed low *in vivo* generation of $OH^{\bullet}$ warrants further studies but is consistent with the effect reported in isolated perfused rat heart where peroxynitrite evoked both vasodilatation and impaired vascular relaxation.[60] Recent *in vitro* studies[48] have shown that the product yields, in the reaction between peroxynitrite and various substrates, have been found to decrease at higher peroxynitrite concentrations and this may play a part in the present results for SaS tumours. However the roles of peroxynitrite and $OH^{\bullet}$ on salicylate hydroxylation are complex and it has been suggested that they may only be resolved by separation of nitrated and hydroxylated products of aromatic compounds. This approach forms part of our ongoing studies. With regard to tumours, it is possible that the relatively high activity of tumour iNOS and low activity of ecNOS play different roles in determining salicylate hydroxylation and oxidative stress to the tumour vasculature. Future studies will aim to distinguish between peroxynitrite-dependent aromatic hydroxylation and that involving free $OH^{\bullet}$, and their relationship to cytotoxicity.

The NOS activity of normal murine skin is very low (0.92 ± 0.14 nmol NO_3^-/100 mg/6 h), and approximately 2-fold less than CaNT and 18-fold less than SaS tumours.[3] Therefore we might have expected skin to display similar responsiveness to I/R injury as CaNT tumours. We are unable to apply the same cytotoxicity assay to skin tissue, as was used for tumour studies, but there was no gross histological evidence of vascular damage at 1 h reperfusion following 1 h vascular occlusion. The differential response of these tissues is evidenced by the increased (hyperemic) response in skin blood flow contrasts with the significant damage evident in the CaNT tumour. Inhibition of skin NOS did not result in significantly increased levels of $OH^{\bullet}$ generation and therefore indicates a negligible role for $NO^{\bullet}$ in skin response. It appears that low $NO^{\bullet}$ production is not the main factor in conferring resistance to oxidative injury and vascular damage by I/R injury in skin. Other factors, such as vascular structure and enzymatic changes during ischaemia are likely candidates to explain the tissue-dependent response. However the present study would suggest that there is a therapeutic window for this type of oxidative injury between skin and tumours which can be increased by inhibition of NOS activity.

The accumulation of neutrophils and their associated oxidative burst has been indicated as mediators of I/R injury in normal tissues.[8] Adhesion and activation of neutrophils is undoubtedly associated with damage to vascular endothelium and may be modulated by $NO^{\bullet}$ and increased by NOS inhibition.[36] In addition, neutrophil oxidative burst has been shown to be critically dependent upon oxygen tension and availability of $NO^{\bullet}$.[61] Following an *iv* injection

of neutrophils, pre-labelled with the fluorescent DNA marker Hoechst 33342, we have shown that the adhesion and infiltration of neutrophils into the reperfused CaNT tumour is significantly greater than the SaS tumour.[62] Since activated neutrophils produce $O_2^{\bullet-}$ via an NADPH-dependent oxidase we intend to investigate the relative contribution of endothelial or neutrophil oxidative burst in determining the generation of $OH^{\bullet}$ and resulting cytotoxicity of tumours following I/R injury.

The present study indicates that generation of $OH^{\bullet}$ occurs soon after release of vascular occlusion and is predictive for the vascular and cytotoxic damage following I/R injury to murine tumours. The production of $NO^{\bullet}$ by tumours is protective against I/R injury and cytotoxicity can be increased if NOS is inhibited. The hyperemic response in normal skin, suggesting minimal vascular damage, combined with low production of $NO^{\bullet}$ implies that $NO^{\bullet}$ is not the sole determinant of I/R injury in skin. Future studies will identify the potential of compromised antioxidant defenses or increased neutrophil–endothelium adhesion molecules in potentiating the therapeutic advantage of vascular and cytotoxic response of tumours to oxidative stress.

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