The Virtual Free Radical School

PEROXYNITRITE, CARBON DIOXIDE, AND FREE RADICAL PRODUCTION

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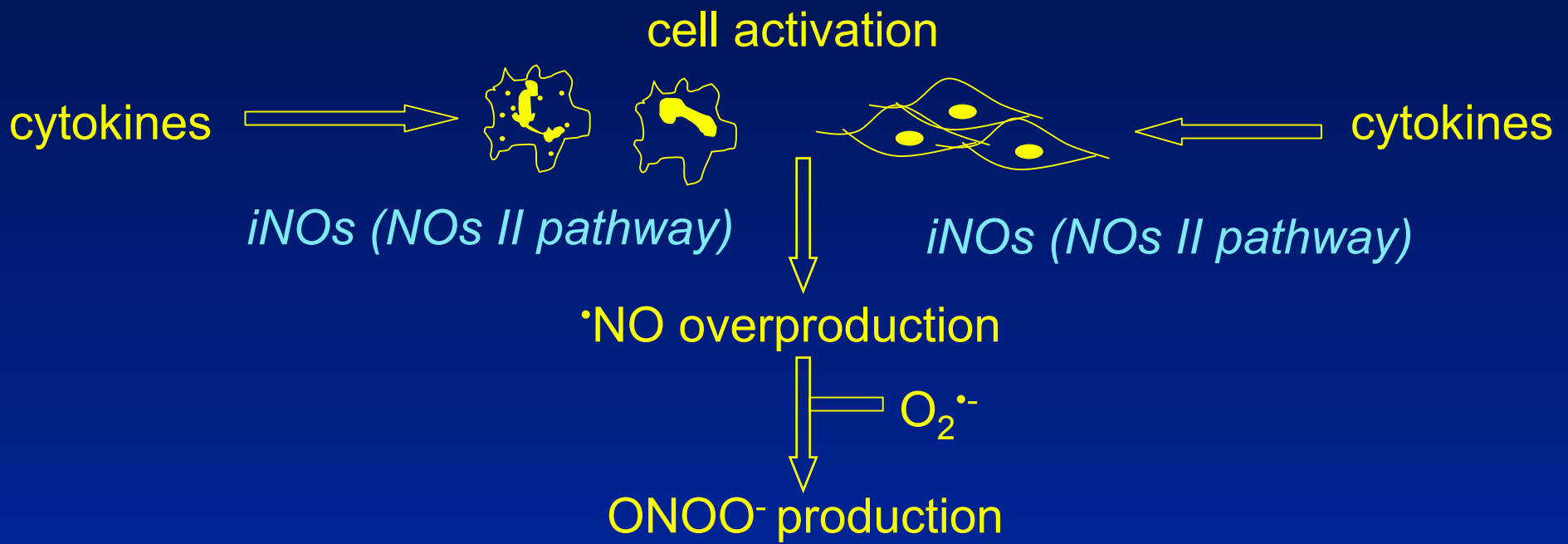
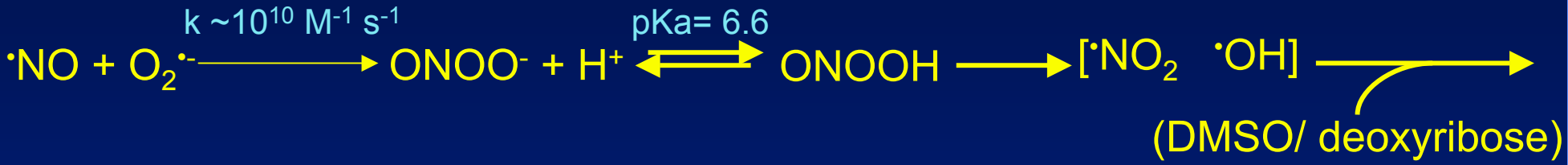
Peroxynitrite production

Although peroxynitrite production *in vivo* remains to be unequivocally demonstrated, the assumption of its formation provides the framework for the understanding of several pathophysiological phenomena such as macrophage microbicidal mechanisms and cell and tissue injury associated with inflammatory conditions. These processes are linked to an overproduction of •NO resulting from the activation of signaling pathways that lead to the expression of the inducible •NO synthase enzyme in several cells and tissues.



Peroxynitrite as a free radical producer

Beckman and co-workers were the first to propose that when overproduced, $^{\bullet}NO$ was likely to react with $O_2^{\bullet -}$ to form peroxynitrite (Beckman *et al., Proc Natl Acad Sci. USA* **87**:1620, 1990). This seminal paper attracted much attention because based on scanty chemical literature and on the oxidation of DMSO and deoxyribose, it proposed that peroxynitrite could be a transition metal ion-independent route for hydroxyl radical production *in vivo.*

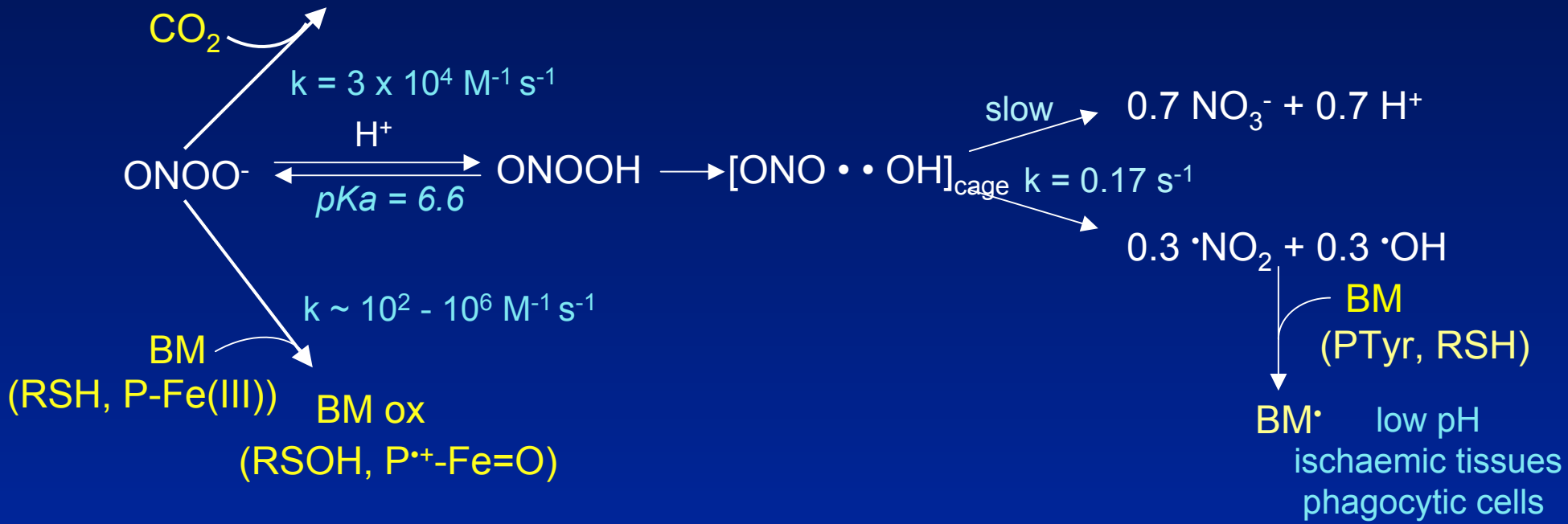


All the attention led to many papers and to a huge controversy about the possibility of free radical production from peroxynitrite *in vitro*. The controversy was recently resolved and the present knowledge of peroxynitrite reactions *in vitro* and in cells indicates that the oxidant is likely to act as free radical producer *in vivo.* Relevant to this behavior is the rapid reaction of peroxynitrite with the biologically ubiquitous CO_2 that increases peroxynitrite--mediated one-electron oxidation and nitration of biomolecules. In some environments, reaction of peroxynitrite with hemproteins may also become important for biomolecule--derived radical production (next slides) (Augusto *et al.*, *Free Rad Biol Med.* **32**:849, 2002).

Peroxynitrite reactions *in vitro*

Proton-catalyzed decomposition *versus* reaction with biotargets

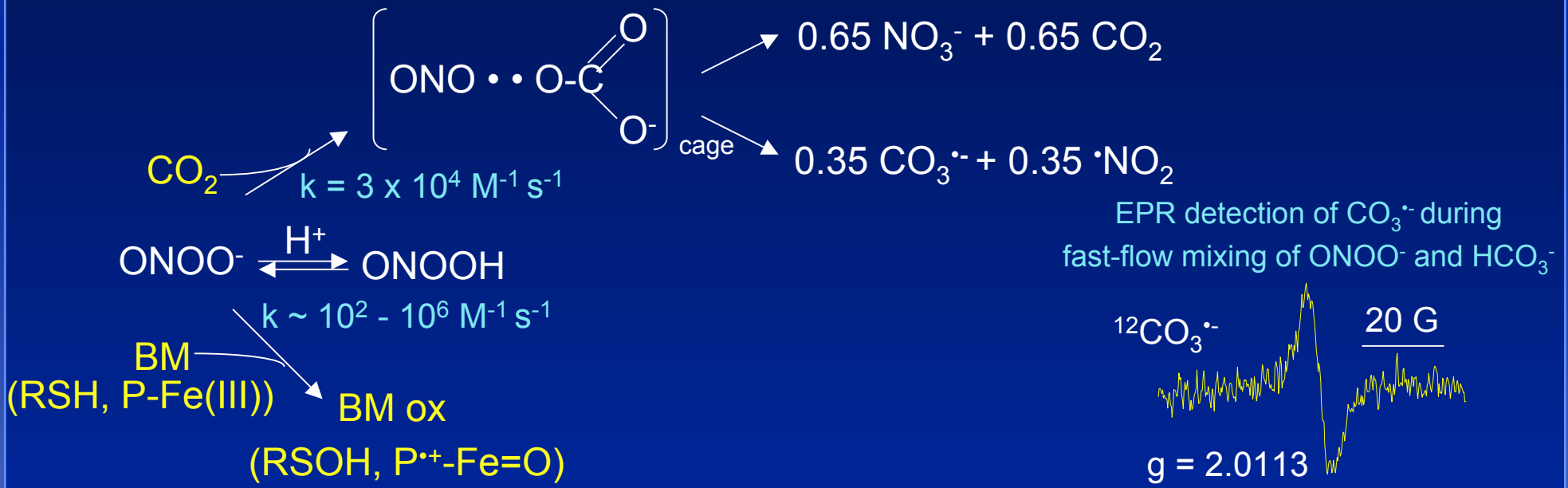
Peroxynitrite is stable at alkaline pHs but upon protonation (pKa= 6.6) it decomposes to yield 70% NO_3^- and 30% $^{\bullet}OH$ and $^{\bullet}NO_2$ that can oxidize biomolecules (BM) to the corresponding radicals. *In vivo,* however, these one-electron oxidations are likely to become relevant only at acid pH because at neutral pH the proton-catalyzed decay is too slow to compete with biotargets such as CO_2, biothiols (RSH) and hemoproteins (P-Fe (III) that react directly with peroxynitrite (calculations to predict dominant routes are shown in slide 8).



Peroxynitrite reactions *in vitro*

Two- *versus* one-electron oxidations

RSH, P-Fe(III) and CO_2 are anticipated to be the most important peroxynitrite biotargets because of their high biological concentrations and rapid reaction rate with the oxidant ($k \sim 10^2$-$10^6\ M^{-1}.\ s^{-1}$). These reactions greatly reduce the half-life of peroxynitrite (from s to ms) and the targets are usually oxidized by two-electron mechanisms. An important exception is the reaction with the biologically ubiquitous CO_2 that produces 65% NO_3^- and 35% $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ (Bonini *et al., J Biol Chem.* **274**:10802, 1999).



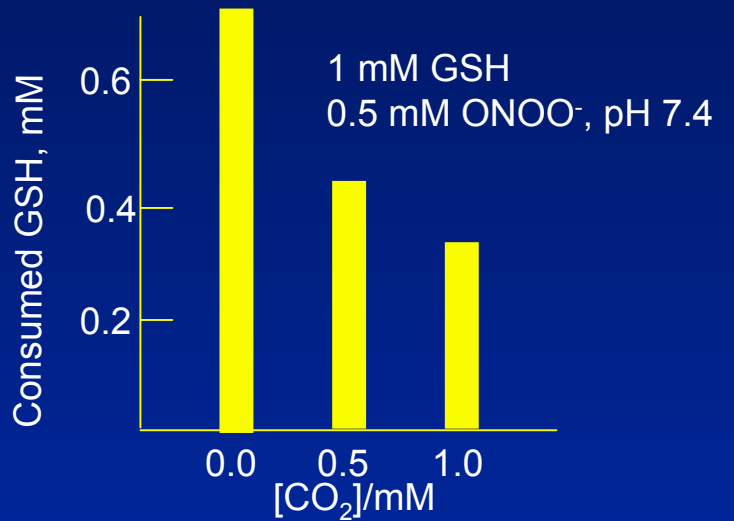
EPR detection of $CO_3^{\bullet-}$ during fast-flow mixing of $ONOO^-$ and HCO_3^-

Peroxynitrite reactions *in vitro*

CO_2 diverts peroxynitrite reactivity from two- to one-electron mechanisms.

The radicals produced from the reaction between peroxynitrite and CO_2, $CO_3^{\bullet-}$ ($E^{o'}$= 1.8 V; pH 7.0) and $^{\bullet}NO_2$ ($E^{o'}$= 0.9 V, pH= 7.0), are oxidizing radicals that can oxidize several biotargets to their corresponding radicals. Then, by competing with other targets for peroxynitrite, CO_2 may divert their oxidation from two- to one-electron mechanisms. This was clearly shown in the case of peroxynitrite-mediated oxidation of biothiols at pH 7.4. For instance,GSH two-electron oxidation is inhibited by CO_2 whereas its one-electron oxidation is increased (below) (Bonini & Augusto, *J Biol Chem.* **276**:9749, 2001).

GSH 2e$^-$ oxidation

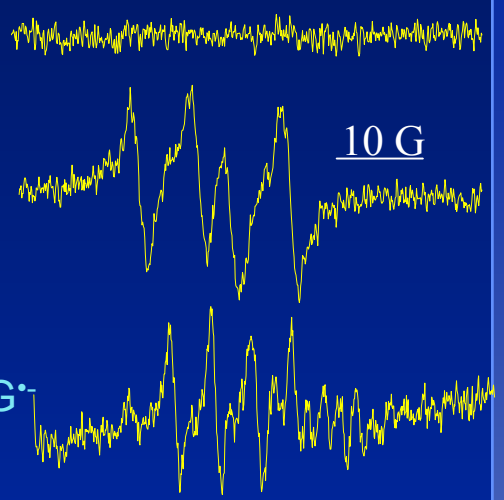


GSH 1e$^-$ oxidation

5 mM $ONOO^-$
+ 5/ 25 mM GSH, pH 7.4
(no radicals are detectable)

5 mM $ONOO^-$
+ 5 mM GSH
+ 5 mM CO_2, pH 7.4
$GSO^{\bullet}$

5 mM $ONOO^-$
+ 25 mM GSH
+ 5 mM CO_2, pH 7.4
$GSSG^{\bullet-}$



Peroxynitrite reactions *in vitro*

CO_2 diverts peroxynitrite reactivity from two- to one-electron mechanisms.

The reported effect of CO_2 in inhibiting GSH two-electron oxidation while increasing its one-electron oxidation at pH 7.4 provides an useful example to understand peroxynitrite reactivity *in vitro.* Taken into account the competing peroxynitrite reactions, the expected yields of GSH two- (GSSG) and one-electron ($GS^{\bullet}$) products were calculated (see, slide 8) and shown to be in good agreement with the experimentally measured yields of consumed GSH (Table I) (Bonini & Augusto, *J Biol Chem.* **276**:9749, 2001).

Table I- Comparison of the experimental yields of consumed GSH with calculated yields of products formed during the oxidation of 1 mM GSH by 0.5 mM peroxynitrite in the presence and absence of 1 mM CO_2 at 25ºC.

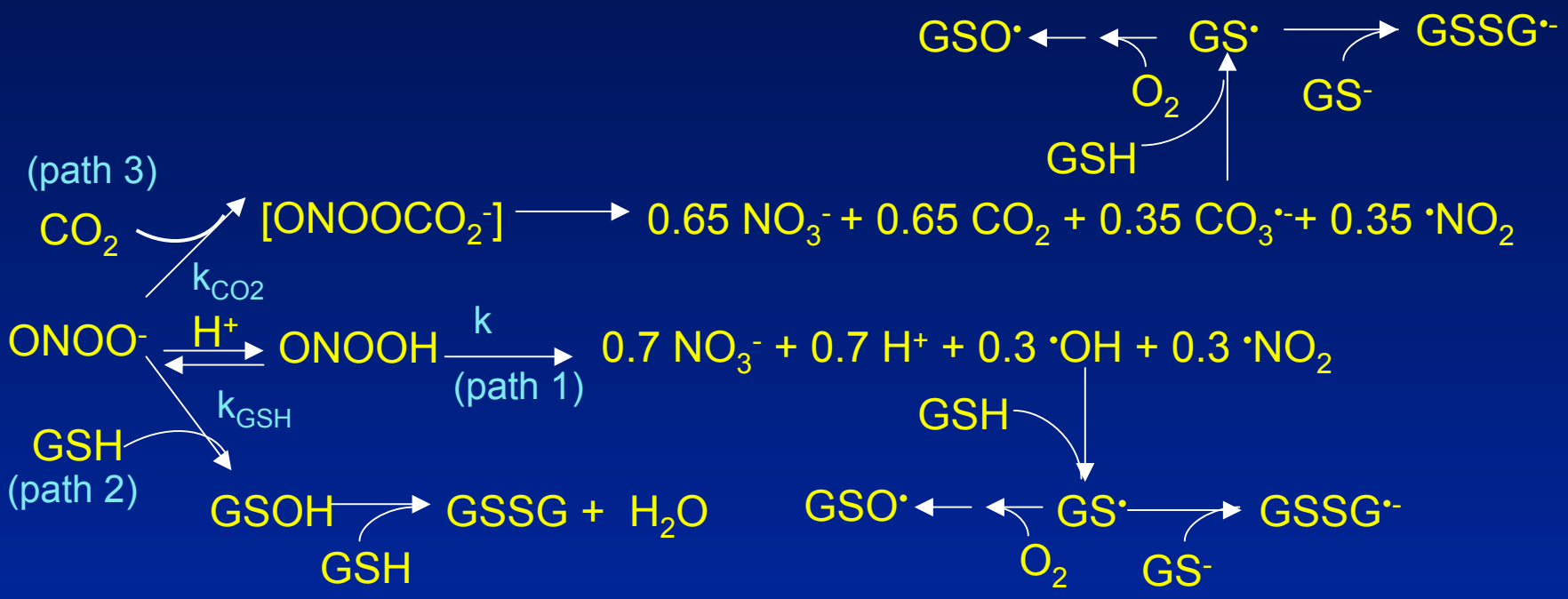
System	Measured Consumed GSH	Calculated product concentrations $^{\bullet}OH + {}^{\bullet}NO_2$ (=$GS^{\bullet}$)	$CO_3^{\bullet-} + {}^{\bullet}NO_2$ (=$GS^{\bullet}$)	GSSG
	mM	mM	mM	mM
GSH, pH 7.4	0.72	0.06		0.80
GSH + CO_2, pH 7.4	0.36	<0.02	0.32	0.02
GSH, pH 5.4	0.26	0.26		0.10
GSH + CO_2, pH 5.4	0.30	0.10	0.20	0.04

Peroxynitrite reactions *in vitro*

CO_2 diverts peroxynitrite reactivity from two- to one-electron mechanisms.

CALCULATIONS I

To calculate the expected product concentrations shown in Table I, the competing peroxynitrite reactions were considered at each experimental condition. The steps used to calculate product yields with 0.5 mM peroxynitrite, 1 mM GSH and 0 or 1 mM CO_2 at pH 7.4, 25°C are detailed next. Recall that rate constants (k) change with pH and temperature.



Peroxynitrite reactions *in vitro*

CO_2 diverts peroxynitrite reactivity from two- to one-electron mechanisms.

CALCULATIONS II

1. The % of peroxynitrite that decays by each of the three competing pathways (path 1, 2 and 3 in slide 8) is calculated with the equation below by substituting the experimental concentrations of GSH (1 mM) and CO_2 (0 or 1 mM) and the known rate constants at pH 7.4 and 25°C (k= 0.17 s^{-1}, k_{GSH}= 6.6 x 10^2 M^{-1} s^{-1} and k_{CO2}= 2.6 x 10^4 M^{-1} s^{-1}).

$$\frac{d[ONOO^-]}{dt} = (k + k_{GSH}[GSH]\, k_{CO2}[CO_2])\,([ONOO^-]$$

2. The calculation shows that in the absence of CO_2, 80 and 20% peroxynitrite reacts with GSH (path 2) and suffer acid-catalyzed decomposition (path 1), respectively. Since 0.5 mM peroxynitrite was used, 0.4 mM GSOH (0.5 x 0.8) should be produced. The latter is unstable and it is likely to react with excess GSH to produce the calculated 0.8 mM GSSG (Table I). With 0.5 mM peroxynitrite, 0.03 mM each $^{\bullet}OH$ and $^{\bullet}NO_2$ should be produced (0.5 x 0.2 x 0.3) and since both radicals can oxidize GSH they produce the calculated 0.06 mM $GS^{\bullet}$ (Table I).

Peroxynitrite reactions *in vitro*

CO_2 diverts peroxynitrite reactivity from two- to one-electron mechanisms.

CALCULATIONS III & CONCLUSIONS

3. The calculation shows that in the presence of 1 mM CO_2, 97, 2 and <1% peroxynitrite reacts with CO_2 (path 3) , GSH (path 2) and suffer acid-catalyzed decomposition (path 1), respectively. With 0.5 mM peroxynitrite, 0.16 mM each $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ should be produced (0.5 x 0.97 x 0.35) and since both radicals can oxidize GSH they produce 0.32 mM $GS^{\bullet}$ (Table I).

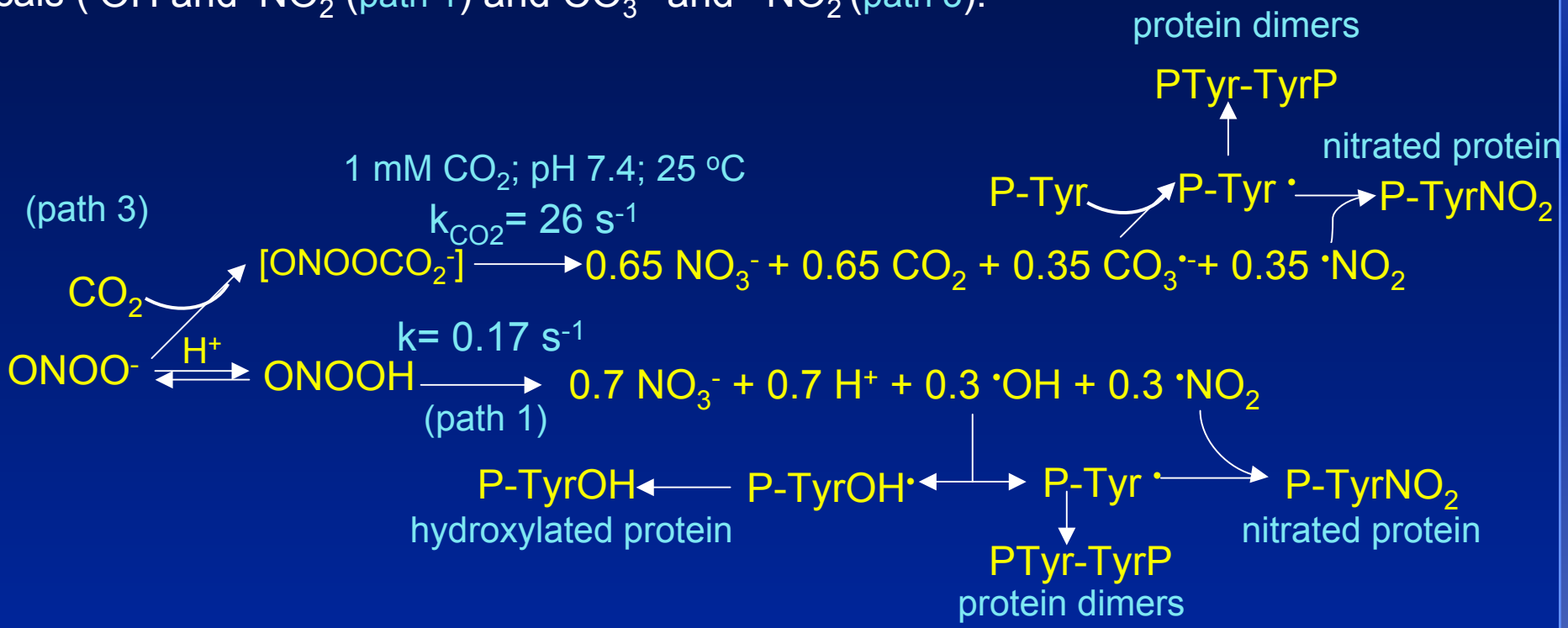
CONCLUSIONS

The results and calculations (Table I) show that at pH 7.4 and in the absence of CO_2, most of GSH is oxidized by peroxynitrite to GSSG *via* two-electrons (path 2) (0.72 and 0.8 mM experimental and calculated, respectively). In the presence of CO_2, total GSH oxidation is inhibited (0.36 and 0.32 mM experimental and calculated, respectively) but most fraction that is oxidized is so by the produced $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ to render GSH-derived radicals ($GS^{\bullet}$, $GSO^{\bullet}$ and/or $GSSG^{\bullet-}$, depending on O_2 and GSH concentration) (path 3). At pH 5.4, GSH oxidation to radicals predominates even in the absence of CO_2 (path 1). It should be emphasized that all of the GSH-derived radicals produced were detected and identified by EPR) (Bonini & Augusto, *J Biol Chem.* **276**:9749, 2001).

Peroxynitrite reactions *in vitro*

CO_2 amplifies peroxynitrite-mediated nitrations

CO_2 also increases peroxynitrite-mediated nitration of tyrosine residues in proteins (P-Tyr) and of guanine residues in nucleotides, RNA and DNA. In these cases, the mechanism is different than the one operating in the case of biothiol oxidation because Tyr and Gua residues do not react directly with peroxynitrite but instead, with its derived radicals ($^{\bullet}OH$ and $^{\bullet}NO_2$ (path 1) and $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ (path 3).



Peroxynitrite reactions *in vitro*

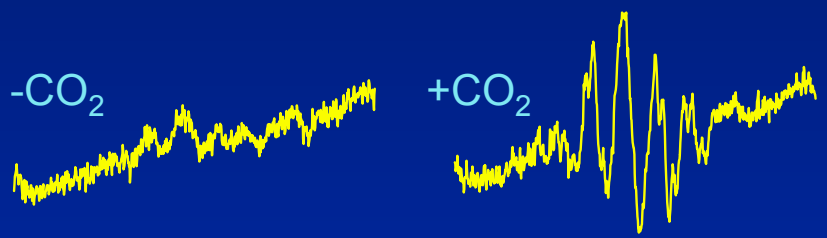
CO_2 amplifies peroxynitrite-mediated nitrations

Still, CO_2 increases several fold the nitrations mediated by peroxynitrite. This effect can be ascribed to:

(i) The more specific reactivity of the $CO_3^{\bullet-}$ as compared with the $^{\bullet}OH$ radical. Being less discriminative, the $^{\bullet}OH$ radical attacks different molecules or different sites in the same molecule producing lower yields of a specific product. In the case of Tyr, the $^{\bullet}OH$ radical can add and oxidize the amino acid producing at least two different products (nitrated and hydroxylated Tyr). In contrast, the $CO_3^{\bullet-}$ only oxidizes Tyr to the Tyr $^{\bullet}$ radical (slide 11).

(ii) The faster radical production rate in the presence of CO_2 that increases radical fluxes and consequently, the possibility of radical-radical recombination such as the one that produces nitrated proteins P-Tyr $^{\bullet}$ + $^{\bullet}NO_2$ ⟶ P-TyrNO_2 (slide 11; compare k values).The effect on radical fluxes can be seen in fast-flow EPR experiments that detected higher Tyr $^{\bullet}$ radical yields in the presence of CO_2 (Santos *et al.*, *Arch Biochem Biophys.* **377**:147, 2000).

EPR detection of Tyr $^{\bullet}$ during fast-flow mixing of $ONOO^-$ with Tyr^-



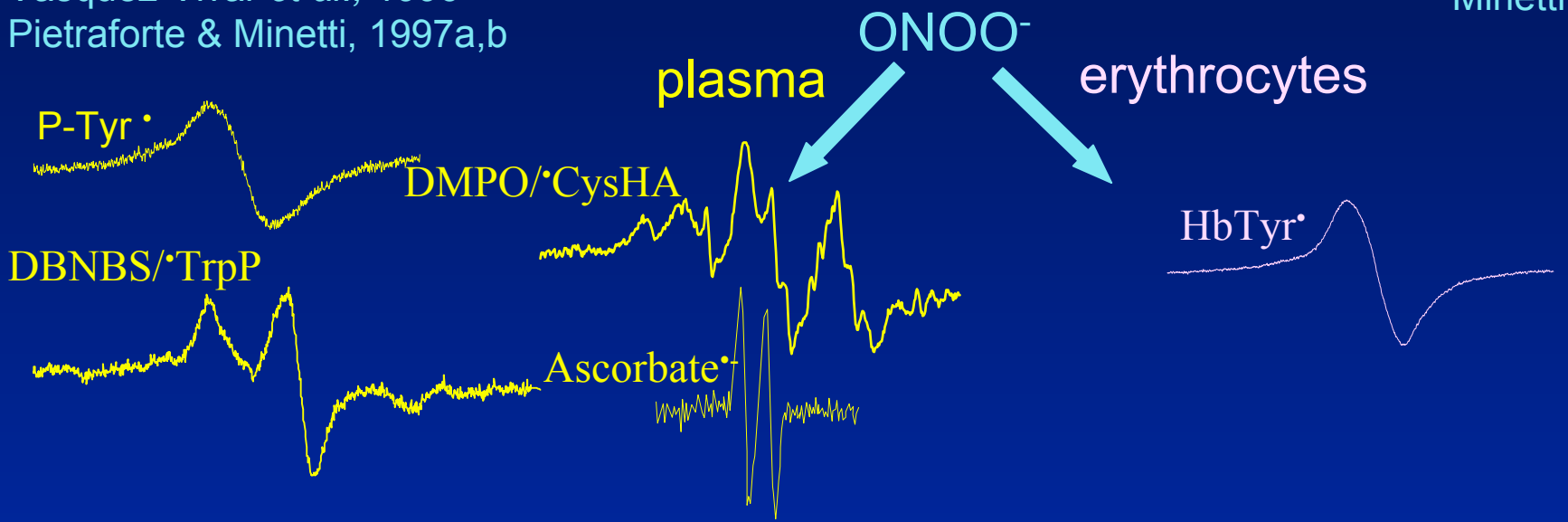
Peroxynitrite reactions in biological fluids and cells

Biomolecule-derived radical production

The concentration of CO_2 in equilibrium with HCO_3^- in intra and extra-cellular fluids is high (1-1.5 mM). Then, in these environments, peroxynitrite is likely to act through its derived radicals ($CO_3^{\bullet-}$ and $^{\bullet}NO_2$) and consequently, as a biomolecule radical producer (at acid pHs, peroxynitrite-derived $^{\bullet}OH$ may also become relevant). In fact, even before peroxynitrite reactivity had been completely elucidated, EPR studies by ourselves and by Minetti's group showed that peroxynitrite addition to plasma and erythrocytes led to the production of several biomolecule-derived radicals (below).

Vasquez-Vivar *et al.*, 1996
Pietraforte & Minetti, 1997a,b

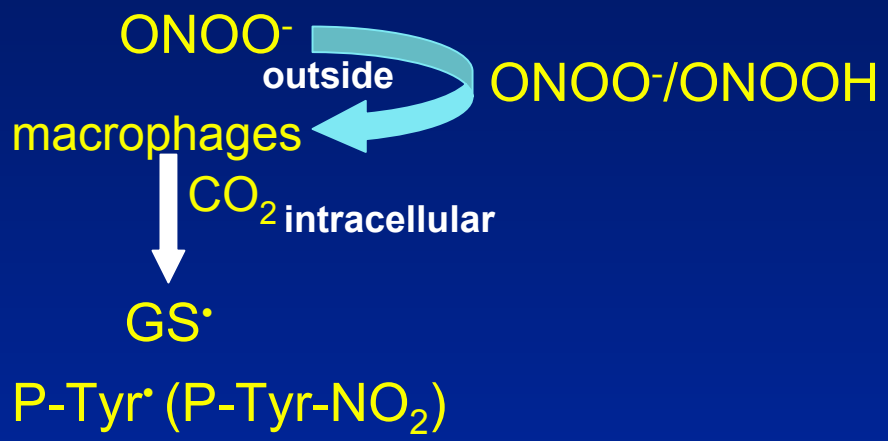
Minetti *et al.*, 1999

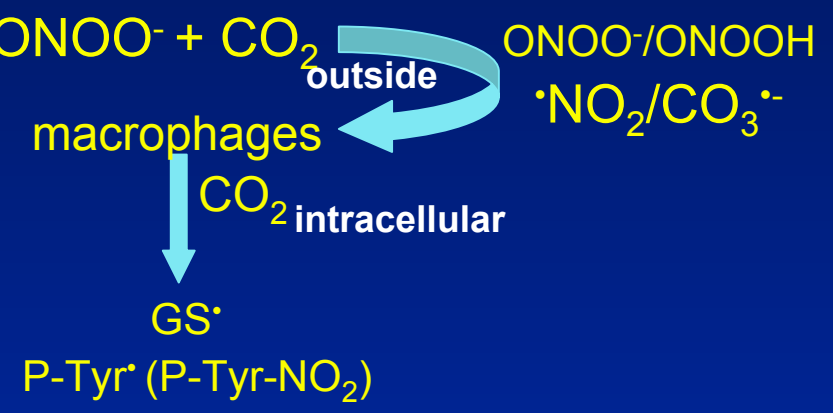


Biomolecule-derived radical production in macrophages

Glutathiyl and protein-tyrosyl radicals

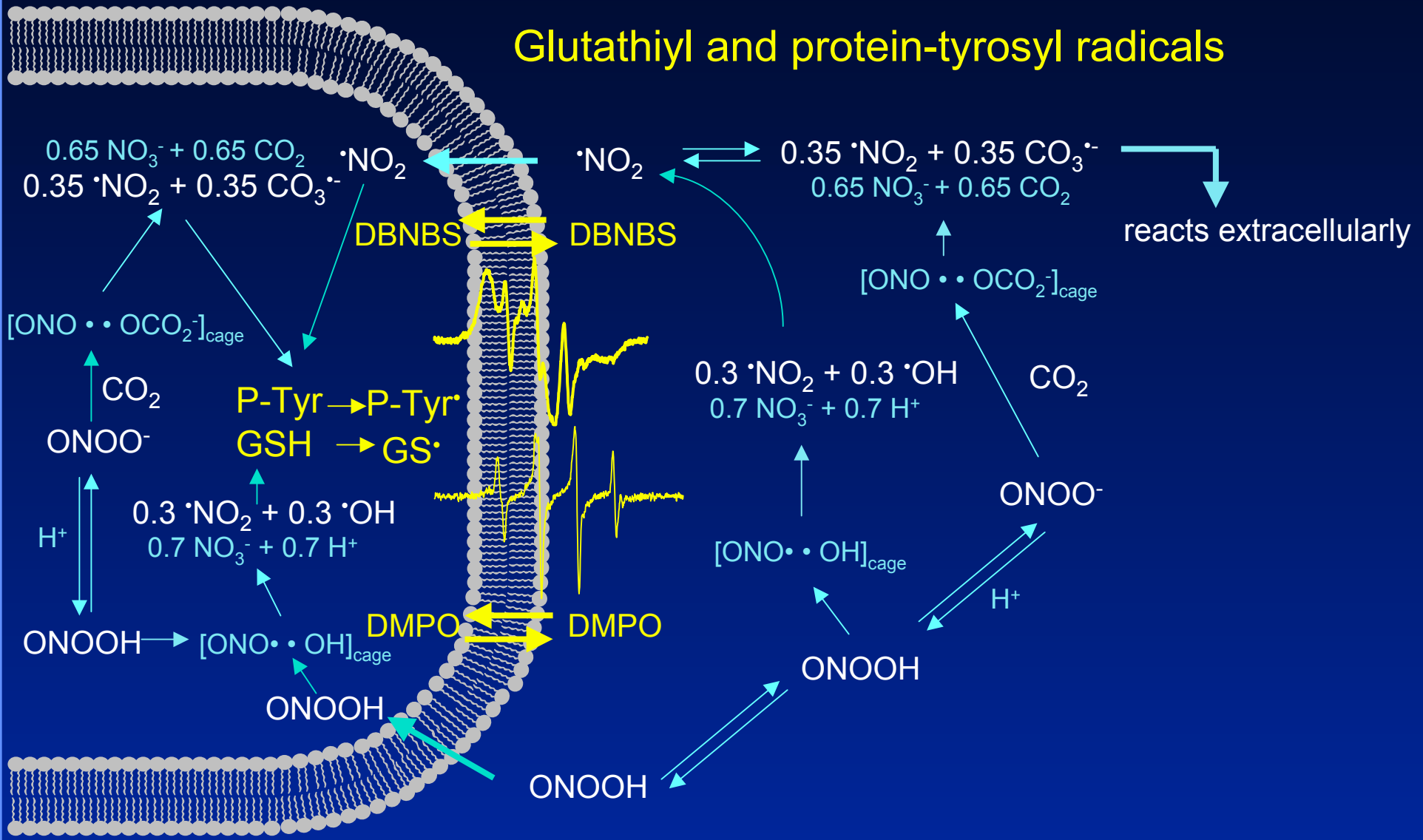
To better understand the production of biomolecule-derived radicals from peroxynitrite in cells, we have studied the interaction of peroxynitrite with macrophages (J774) (Menezes & Augusto, *J Biol Chem.* **276**:39879, 2001) by EPR spin trapping and western blotting. The results indicate that GSH and P-tyrosine residues are the main targets of peroxynitrite and that they are oxidized to the corresponding radicals *via* intracellular CO_2 (below left). Addition of exogenous CO_2 inhibited radical yield because the amount of oxidant that diffuses into the cells decreases (below right). $ONOO^-$, ONOOH and $^{\bullet}NO_2$ can permeate cell membranes but the charged $CO_3^{\bullet -}$ cannot (detailed view, slide 15) (Augusto *et al.*, *Free Rad Biol Med.* **32**:849, 2002).





Biomolecule-derived radical production in macrophages

Glutathiyl and protein-tyrosyl radicals

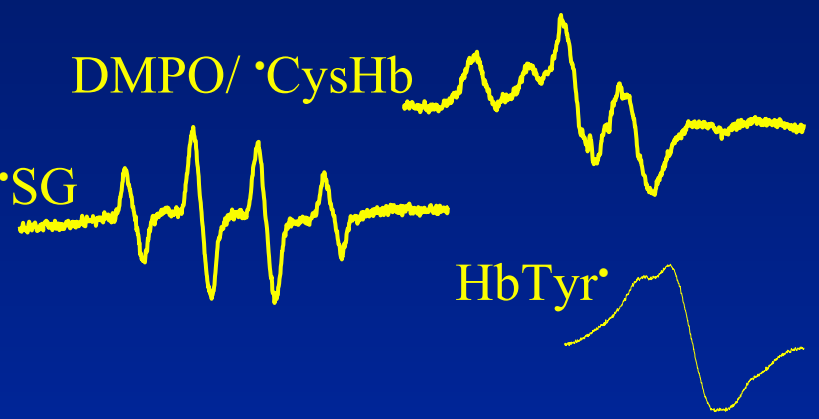


Biomolecule-derived radical production in erythrocytes

Glutathiyl, hemoglobin-thiyl and hemoglobin-tyrosyl radicals

Treatment of fibroblasts with peroxynitrite also led to results similar than those obtained with macrophages (Menezes, 2002, Masters Dissertation). This indicates that GSH and protein-tyr residues are likely to be the important targets of peroxynitrite in most cells and that they are oxidized to the corresponding radicals by peroxynitrite produced both extracellularly and intracellularly through CO_2 intermediacy (slide 15) (Augusto *et al.*, *Free Rad Biol Med.* **32**:849, 2002). The situation changes in the case of erythrocytes because peroxynitrite should react mostly with oxyhemoglobin rather than with CO_2 (both react with peroxynitrite with similar rate constants, *c.a.*10^4 M^{-1} s^{-1}, but oxyhemoglobin concentration is higher (*c.a.* 20 mM *versus c.a.*1.5 mM). Still, the $GS^{\bullet}$ is produced together with Hb-$Cys^{\bullet}$ and Hb-$Tyr^{\bullet}$ (Augusto *et al.*, 2002, *Biochemistry,* in press).

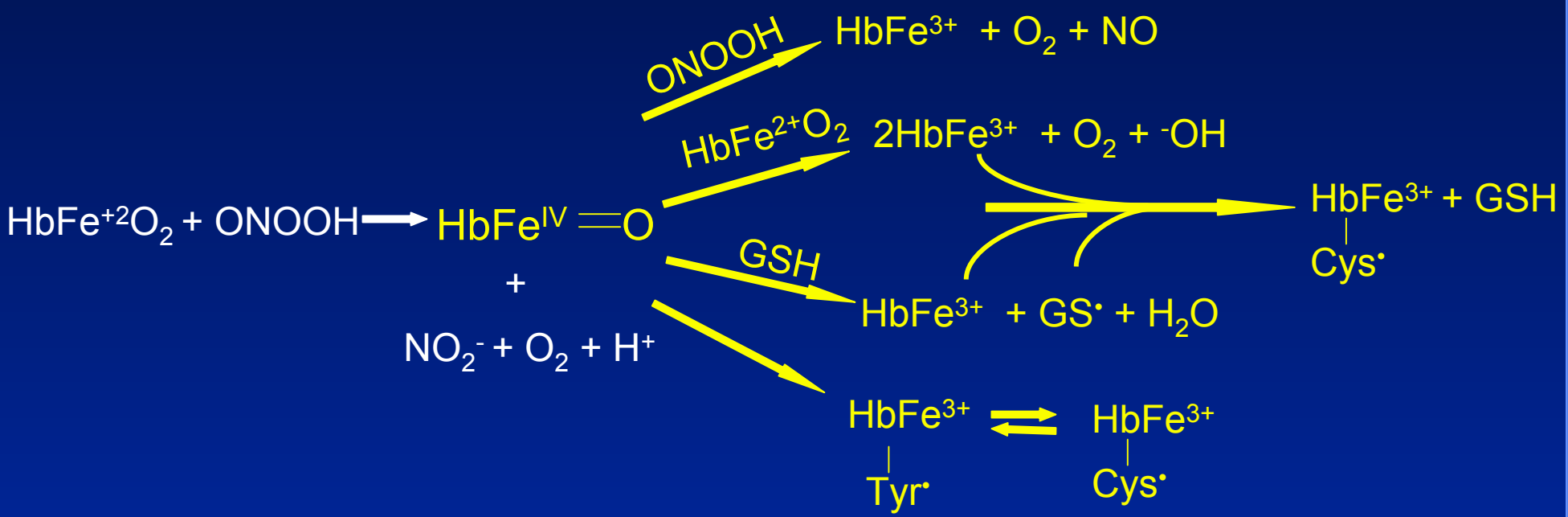
$ONOO^-$ ⟹ erythrocytes ⟹ DMPO/ $^{\bullet}$SG



Biomolecule-derived radical production in erythrocytes

Glutathiyl, hemoglobin-thiyl and hemoglobin-tyrosyl radicals

Our studies of the interaction of peroxynitrite with human erythrocytes taken together with published findings indicate that oxyhemoglobin is first oxidized to ferryl-hemoglobin. This species decays by several routes, including the oxidation of intracelluar GSH to the GS•, and oxidation of its own amino acid residues to produce Hb-Cys• and Hb-Tyr • (Augusto *et al.*, 2002, *Biochemistry,* in press).



Peroxynitrite reactions in biological fluids and cells

Production of glutathiyl, protein-thiyl and protein-tyrosyl radicals

The EPR studies summarized in slides 13-17, demonstrate that addition of peroxynitrite to biological fluids and cells produce biomolecule-derived radicals.

Cells-In all studied cells, the $GS^{\bullet}$ radical was detected. This radical appears to be produced mainly through GSH oxidation by the radicals produced from the reaction of peroxynitrite with intracellular CO_2 ($CO_3^{\bullet-}$ and $^{\bullet}NO_2$). In a special cell-type such as erythrocytes, the ferryl-hemoglobin resulting from the reaction between peroxynitrite and oxyhemoglobin oxidizes GSH to $GS^{\bullet}$. In parallel with $GS^{\bullet}$, P-Tyr $^{\bullet}$ was produced in macrophages and fibroblasts whereas both Hb-Cys$^{\bullet}$ and Hb-Tyr $^{\bullet}$ were produced in erythrocytes. As expected from P-Tyr $^{\bullet}$ production, nitrated proteins were detected in all studied cells.

Plasma-The studies with cells indicate that the previously detected biomolecule-derived radicals in human plasma treated with peroxynitrite (Ascorbate$^{\bullet-}$, Albumin-S$^{\bullet}$, Albumin-Tyr $^{\bullet}$, Albumin-Trp$^{\bullet}$, P-Tyr $^{\bullet}$ and Protein-Trp$^{\bullet}$) were produced mainly through the intermediacy of plasma CO_2.

Peroxynitrite as a biomolecule-derived radical producer

SUMMARY

(i) Peroxynitrite is likely to act through its derived radicals ($CO_3^{\bullet-}$ and $^{\bullet}NO_2$) *in vivo* due to the high concentration of CO_2 in equilibrium with HCO_3^- in most biological fluids. In low pH environments, the peroxynitrite-derived $^{\bullet}OH$ radical can also become relevant.

(ii) Both, $CO_3^{\bullet-}$ ($E^{o'} = 1.8$ V; pH 7.0) and $^{\bullet}NO_2$ ($E^{o'} = 0.9$ V, pH = 7.0) are oxidizing radicals that can oxidize several biotargets to their corresponding radicals. A flux of both radicals (as produced from peroxynitrite) is very efficient in nitrating tyrosine and guanine residues by combining the oxidizing power of $CO_3^{\bullet-}$ with the radical recombination reactions of $^{\bullet}NO_2$.

(iii) The main biomolecules that are likely to be oxidized to their corresponding radicals by peroxynitrite-derived radicals *in vivo* are GSH, P-SH, P-Tyr, P-Trp, RNA-Gua and DNA-Gua.

(iv) Oxidation of GSH to $GS^{\bullet}$, occurs even in environments where peroxynitrite reacts preferentially with hemoproteins rather than with CO_2 . Then, oxidation of GSH to $GS^{\bullet}$ is likely to be a consequence of peroxynitrite production *in vivo*.

Augusto *et al.*, *Free Rad Biol Med.* **32**:849, 2002