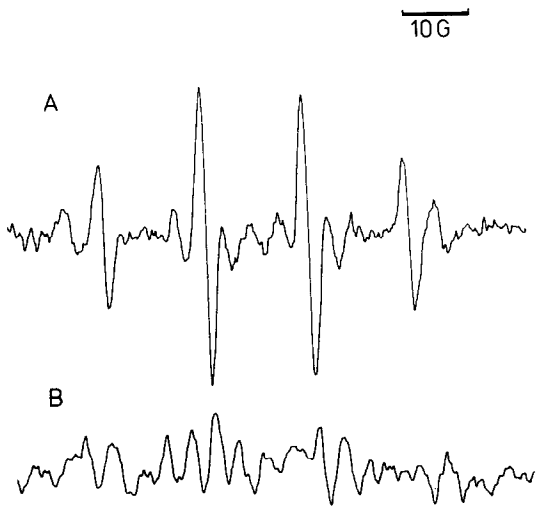

Fig. 2. ESR spectra obtained from mixtures of $MPDP^+$ and MPP^+. $MPDP^+$ and MPP^+, 10 mM each, were incubated in the presence of 90 mM DMPO in phosphate buffer containing DETAPAC, pH 7.4 (A) and in the presence of SOD, 25 μg/ml (B). See legend of Fig. 1 for other parameters.

DMPO. These experiments indicate that a redox reaction occurs between MPP^+ and $MPDP^+$, during which an electron is transferred to oxygen to form superoxide. The following reaction is suggested:

$$(a)\ MPP^+ + MPDP^+ <---> 2\,MPP\cdot + 2\,H^+$$

$$(b)\ 2\,MPP\cdot + 2\,O_2 <---> 2\,O_2^- + 2\,MPP^+$$

In summary, incubation of MPTP with mitochondria from brain resulted in oxygen-dependent formation of free radicals. Using ESR, the spin adduct of hydroxyl radical was detected. SOD suppressed the formation of radical, identifying it as superoxide. Deprenyl, an MAO B inhibitor, diminished the generation of the ESR signal, suggesting that the generation of free radicals is MAO B dependent. An ESR spectrum was obtained when the metabolites of MPTP, $MPDP^-$ and MPP^+, were incubated together in the absence of mitochondria. The formation of free radicals during the biotransformation of MPTP in brain may play a key role in its neurotoxic activity.

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Hydroxylation of aniline mediated by heme-bound oxy-radicals in a heme peptide model system

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It has been documented that heme containing proteins such as hemoglobin and myoglobin can substitute cytochrome-P450 in hydroxylation and demethylation reactions *in vitro* due to their ability to form reactive oxy-radicals during the autoxidation of their heme [1–5]. It has also been found that heme-nonapeptide (HP), a proteolytic fragment of cytochrome *c* is a good substrate of NADPH : cytochrome-P450-reductase [6]. Its inhibitory effect on lipid peroxidation in rat liver and brain microsomes [7–9] was explained by its capability of shunting electrons from microsomal reductases and/or of binding and eliminating the oxy-radicals [6].

In the widely accepted reaction scheme of drug metabolism, cytochrome-P450 is reduced by specific reductases and reoxidized by O_2. In addition, cytochrome-P450 binds and eliminates oxy-radicals [10]. Our earlier results with

HP (see Refs [6–9]) suggest that HP might behave similarly to cytochrome-P450 in many aspects. In order to further establish such a similarity, aniline hydroxylation catalyzed by HP was investigated and the findings are reported here.

Materials and methods

HP was purchased from Reanal Fine Chemicals, Budapest, Hungary, glucose-6-phosphate dehydrogenase, superoxide dismutase and catalase from Serva. All the chemicals used were of the highest purity available. NADPH:cytochrome-P450-reductase (EC 1.6.2.4) was prepared from detergent solubilized microsomes of phenobarbital induced rat liver according to [11]. The activity of the purified reductase was about 60 units/mg protein as assayed by monitoring the NADPH-dependent reduction of cytochrome *c*.

Potassium superoxide stock solution was prepared as described by Valentine and Curtis [12].

The standard reaction mixture for aniline hydroxylation contained 40 mM aniline, and 20 μM HP in 0.3 M Chelex-pretreated potassium phosphate buffer (pH 7.5). In order to determine aniline hydroxylation induced by oxy-radicals, the reaction was started by pumping potassium superoxide solution or H_2O_2 into the reaction mixture at a rate of 10 μL solution/min, until they reached the final concentration of 0.5 mM. To measure the reductase-mediated hydroxylation, the reaction was started by the addition of 0.1 U/ml reductase to a mixture also containing 250 μM NADPH plus NADPH regenerating system (6 mM glucose-6-phosphate and 0.5 U/ml glucose-6-phosphate dehydrogenase). The reactions were stopped by the addition of ice-cold trichloroacetic acid.

p-Aminophenol formation was measured as described in [13]. Protein content of the samples was determined according to Wang [14].

Results and discussion

The fact that the Soret peak in the HP spectra shifted from 397 nm to 407 nm upon addition of aniline (Fig. 1) clearly indicated an interaction between HP and aniline. As shown in the insert the K_d, characteristic to the above interaction, is about 2.4 mM and it is similar to that obtained for cytochrome-P450 [15] when binding aniline, a Type II ligand. HP was found to be capable of catalyzing the hydroxylation of aniline in the presence of NADPH:cytochrome-P450-reductase (see Figs 2 and 3).

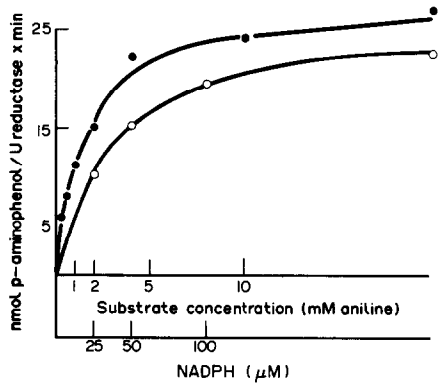

Fig. 2. Substrate concentration-dependence of the rate of aniline hydroxylation. (○——○) varying NADPH concentration at constant aniline (40 mM), (●——●) varying aniline concentration at constant NADPH (250 μM). Other experimental details are described in Materials and Methods.

As seen in Fig. 2, the rate of HP-mediated hydroxylation increased in a saturable fashion by increasing either NADPH or aniline concentration. The apparent K_m values were 40 μM and 1.2 mM for NADPH and aniline, respectively, at saturating HP concentration (20 μM). It should be mentioned that the NADPH consumption was higher than expected for stochiometric aniline hydroxylation (data not shown) suggesting that side reactions also took place (for Ref. see [6]). In order to characterize further the HP mediated aniline hydroxylation, the effects of superoxide dismutase (SOD) and catalase were also investigated. As Fig. 3 shows, *p*-aminophenol formation mediated by HP increased in the presence of SOD and was inhibited by the addition of catalase. These findings could be explained by the fact that the two-step reduction of HP in the presence of oxygen leads to the formation of both O_2^- and H_2O_2 either in free [16] or heme-bound forms [6].

Therefore, addition of SOD results in enhanced production of H_2O_2 by dismutating O_2^- that dissociates from the one-electron-reduced HP–oxygen complex. On the

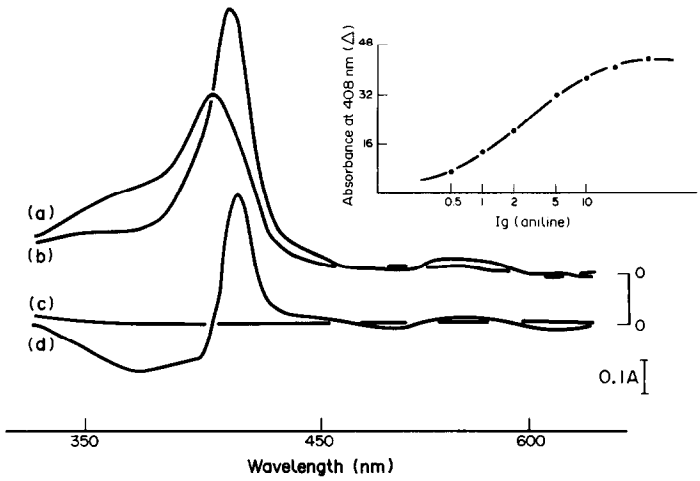

Fig. 1. Aniline induced spectral change of HP. The medium contained 0.3 M potassium phosphate buffer, pH 7.5, and 20 μM HP in spectrum (a) and 20 μM HP + 40 mM aniline in spectrum (b). Spectrum (d) indicates the difference spectrum derived by subtracting spectrum (a) from spectrum (b). Insert: Concentration-dependence of aniline induced absorbance-difference in the HP spectrum at 408 nm.

Table 1. Effect of CO on *p*-aminophenol formation in the presence of 20 μM HP and 40 mM aniline. Experimental details are given in Materials and Methods. Bubbling of gas-mixtures (CO, O_2, N_2) was started 2 min before starting the experiment and was continued during the reaction period.

CO content	Hydroxylation of aniline*	
	NADPH + reg. system	H_2O_2
80% N_2 + 20% O_2	20.75 ± 0.75	26.4 ± 1.6
80% CO + 20% O_2	8.75 ± 0.65	23.8 ± 0.5
100% CO	1.80 ± 0.20	24.1 ± 0.25

* *p*-aminophenol, nmol/min × U EC 1.6.2.4 or nmol/min × μmol H_2O_2 resp.

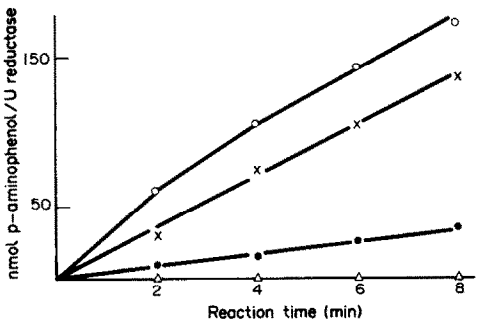

Fig. 3. Effect of SOD and catalase on the HP-mediated, NADPH:cytochrome-P450-reductase catalysed *p*-aminophenol formation. The reaction mixture contained 0.3 M potassium phosphate, pH 7.5, 20 μM HP, 0.1 U/ml reductase, 250 μM NADPH + regenerating system and 40 mM aniline (×——×). Additions: (●——●), 300 U/ml catalase; ○——○, 300 U/ml SOD. △——△ same as ×——× but no HP added.

other hand, catalase could eliminate H_2O_2, a product of the two-electron reduction of HP. This hypothesis suggests that HP catalyses aniline hydroxylation by a reaction mechanism similar to that proposed for cytochrome-P450 (see Ref. [10]).

The similarity between the action of HP and cytochrome-P450 is further supported by the findings (see Table 1) that HP mediates aniline hydroxylation in the presence of H_2O_2 (and in the absence of the reductase) the same way as cytochrome-P450 does [17]. Table 1 also shows that carbon monoxide inhibits reductase-dependent, HP-mediated aniline hydroxylation. As seen, a four-fold excess of carbon monoxide over O_2 results in a higher than 50% inhibition of the hydroxylation reaction similar to that reported for cytochrome-P450 [18].

In conclusion, HP is capable of mediating the hydroxylation of aniline to *p*-aminophenol in the presence of either NADPH plus NADPH:cytochrome-P450-reductase or hydrogen peroxide. In addition, the effects of carbon monoxide, catalase and SOD on the HP-mediated reaction suggest that the mechanism of the HP-catalyzed aniline hydroxylation might be similar to that of cytochrome-P450. Therefore, HP could be a useful tool in studying and modelling the reaction mechanism of cytochrome-P450.

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Attenuation by *N*-ethylmaleimide treatment of the cholinergically induced shortening of action potential duration in guinea pig right atrium

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Parasympathetic stimulation of the heart evokes, among other responses, a rapid and profound bradycardia [1]. Such a response now appears to be mediated, in part, by a cholinergically induced increase in K^+ efflux specifically from the atrial myocardium [2]. An initial clue as to the underlying cellular mechanism was the observation that this hormone-stimulated efflux could be mimicked by the intracellular application of GTP* analogues [3, 4]. These findings thus implicated a role for a guanine nucleotide binding (G) protein in the hormone signalling process, analogous to the involvement of stimulatory and inhibitory G-proteins in the hormonal mediation of adenylyl cyclase [ATP pyrophosphate-lyase (cycling), EC 4.6.1.1]. In support of this hypothesis, it was reported that pertussis toxin blocks the cholinergic activation of this atrial K^+ efflux [4–6]. Furthermore, re-addition of an activated G_i-like protein to such a blocked pathway restores hormonal sensitivity [7]. Despite these conceptual advances, the exact nature of this channel-associated heteromeric G_i-like protein or the active subunit constituent still remains controversial.

Biochemical characterization of the G-protein linked signalling pathway mediating inhibition of cardiac adenylyl cyclase reveals that the integrity of this mechanism is disrupted by alkylation of sulfhydryl (-SH) groups associated with one or more of the pathway components [8]. Decreases in both agonist binding and the degree of cholinergic inhibition have been noted. More specifically, NEM blocks the pertussis toxin catalyzed ADP-ribosylation of G_i-like proteins [9, 10].

In this study, we used NEM as a probe to examine whether the cholinergic signalling mechanism mediating shortening of action potential (AP) duration in mammalian cardiac right atrium involved critical -SH groups. The results indicate that treatment with NEM interfered with cholinergic attenuation of AP duration, through what appears to be disruption the G-protein(s)/cholinergic receptor interaction, as judged by changes in agonist binding characteristics.

Methods

Guinea pigs (male, 300–400 g) were stunned by a sharp blow to the head, followed by cervical dislocation. The hearts were quickly removed and placed in oxygenated (95%/5% O_2/CO_2) HEPES-buffered Tyrode's solution of the following composition (in mM): 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 0.33 NaH_2PO_4, 11.9 $NaHCO_3$, 5 HEPES, 11 dextrose; pH adjusted to 7.3–7.4 with 1 M HCl. Right atria were dissected from the heart and longitudinal strips of muscle (trabeculae) were isolated. Atrial strips were pinned to the Sylgard resin base of a recording chamber (volume = 3 ml) and allowed to equilibrate at least 30 min under low frequency (≈1 Hz) stimulation. Chamber perfusion was ≈1 ml/min.

Preparations were stimulated (Grass S88 stimulator) at one end, using bipolar electrodes, by square wave pulses (1.5–2.5 msec, 1.5–3 V, 2–3 Hz). Action potentials were recorded by a single intracellular microelectrode (tip resistance = 15–30 MΩ) filled with 3 M KCl; a Ag:AgCl agar bridge was used as the indifferent electrode. The recording electrode was connected to a Dagan 8500 preamplifier, and the action potentials and their first derivatives were displayed on a Tektronic 5111 storage oscilloscope and then photographed.

Competitive radioligand binding studies using rabbit atrial homogenates were carried out as previously described [11]. Intact atria (0.2–0.3 g) were incubated for 25 min in oxygenated Tyrodes' solution containing 100 μM NEM and then homogenized in cold 10 mM $NaKPO_4$ buffer at pH 7.4 (10%, w/v) using a PT-10 Polytron. Assay incubations were carried out for 1 hr at 25° in a 1-ml volume containing 10 mM $NaKPO_4$ at pH 7.4, ≈175 pM [^{3}H](−)QNB, competing drugs, guanine nucleotides, and 150–200 μg of frozen homogenate protein. Non-specific binding was determined in the presence of 1 μM atropine sulfate. Samples were rapidly filtered through GF/C filters using a Brandell Cell Harvestor, the filters were then washed with two 5-ml vol. of cold 10 mM $NaKPO_4$ at pH 7.4, and dried overnight, and the trapped radioactivity was counted in 8 ml of a toluene base scintillation mixture at an efficiency of 35–40%.

Results

Cholinergic agonists were observed to produce marked (>50%) shortening of AP duration in stimulated guinea pig right atrial strips (Fig. 1A); other parameters of the AP (resting potential, action potential amplitude, maximal upstroke velocity) were affected only minimally (see Table 1). The onset of the cholinergic effect was noted as early as 1 min after the start of drug superfusion, and the maximal effect was typically observed after 5–7 min. Oxotremorine was found to be the most potent, being approximately 6-fold greater than carbachol and more than 10-fold greater than acetylcholine. (The low potency for acetylcholine was

* Abbreviations: GTP, guanosine 5′-triphosphate; G_i, inhibitory guanine nucleotide binding protein; G_o, guanine nucleotide binding protein of unknown function; Gpp(NH)p, guanosine 5′-(βγ-imino)-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; NEM, *N*-ethylmaleimide; and QNB, quinuclidinyl benzilate.