SUBSTANCE P (SP₁₋₁₁) was exposed to a continuous flux of superoxide (O₂⁻) or hydroxyl radicals (·OH) in a hypoxanthine (HX)/xanthine oxidase (86 mU) system in the presence of 1 mM deferoxamine and 40 mM D-mannitol or 50 μM FeCl₃·6H₂O and 50 μM EDTA, respectively. O₂⁻ caused fragmentation between the Phe² and Phe³, whereas ·OH induced cleavage also between the Phe⁶ and Gly⁹. Reactive oxygen species H₂O₂ and HClO did not cause fragmentation, but modification of the amino acid side chains and/or aggregation with altered hydrophobicity in reverse phase high performance liquid chromatography compared to native SP₁₋₁₁. Furthermore, exposure of SP₁₋₁₁ to phorbol myristate acetate preactivated neutrophils resulted in products similar to those observed upon exposure to superoxide or hydroxyl radicals in a cell-free HX/xanthine oxidase system. This study suggests that, in contrast to rigid proteins, fragmentation is relatively easily induced in a small peptide like SP₁₋₁₁, perhaps due to strain on the peptide and α-carbon bonds caused by the movable, random coil configuration acquired by SP₁₋₁₁ in an aqueous solution. Oxidative modification might modulate paracrine actions of SP₁₋₁₁ at site of inflammation.

Key words: Reactive oxygen species, Substance P

Introduction

Substance P (SP₁₋₁₁) is an undecapeptide, which consists of Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂. SP₁₋₁₁ has been localized to small unmyelinated, slowly conducting C-type polymodal nociceptors. In addition to its possible role in pain transmission in the dorsal horn of the spinal cord, it has been implicated in neurogenic inflammation. Local release by axon reflex as a result of antidromic conduction causes wheal and flare, but various paracrine effects such as synthesis and secretion of type I matrix metalloproteinase (MMP-1 or ‘fibroblast-type’ interstitial collagenase) and prostaglandin E₂ (PGE₂) by synoviocytes, and of interleukin-1β and tumour necrosis factor-α by monocytes, have also been described. In addition to its role in vasoregulation and cytokine and proteinase secretion, substance P can activate polymophonuclear neutrophils (PMN) and monocyte/macrophages to produce superoxide (O₂⁻) via the NADPH oxidase pathway. In many inflammatory diseases hypoxia-reperfusion syndrome is another and possibly more important source of oxygen derived free radicals (ODFR). During ischaemia xanthine dehydrogenase is proteolytically cleaved to xanthine oxidase by an enzyme activated by Ca²⁺ efflux from mitochondria. At the same time, chemical energy from adenosine triphosphate (ATP) is utilized and hypoxanthine (HX) is produced. During reperfusion xanthine oxidase will catalyse the conversion of HX to xanthine and further to uric acid (Equation 1, see below), with bimolecular oxygen acting as an electron acceptor in both reactions. It seems, therefore, that SP₁₋₁₁, released at a site of inflammation, will be exposed not only to degradative enzymes but also to various reactive oxygen species. This prompted us to study the possible effect of such compounds on substance P in vitro.

Materials and Methods

Preparation of synthetic substance P: Synthetic substance P was first purchased from Sigma Chemical Company (St Louis, MO, USA) or Cambridge Research Biochemicals (Cambridge, UK). Because relatively large amounts were needed, substance P was also synthesized according to the solid-phase method with Applied Biosystems (Foster City, CA, USA) 430A peptide synthesizer using tBoc chemistry and p-methyl-BHA-resin.
Production of oxygen-derived free radicals: Synthetic soluble substance P (1 mg/ml) in RPMI-1640 (Gibco) was exposed to $O_2^-$ or hydroxyl radical ('OH) produced in a hypoxanthine/xanthine oxidase (EC 1.2.3.22) system in the presence of 1 mM deferoxamine (Desferal®, Ciba Pharmaceutical Co) and 40 mM $\alpha$-mannitol or 50 $\mu$M FeCl$_3$·6H$_2$O (Mallinckrodt, Paris, KY, USA) and 50 $\mu$M ethylenediaminetetraacetic acid (EDTA) (Sigma) respectively, as described in detail elsewhere. In the presence of these reagents, alterations observed are caused by $O_2^-$, although H$_2$O$_2$ is also formed in a so-called one-electron auto-oxidation or dismutation (Equation 2). Iron was added as iron(III) ions, so that the reaction would start immediately after addition of xanthine oxidase: in this case, iron(III) ions are first reduced to iron(II) ions (Equation 3). Iron(II) ions can then act as an electron donor and catalyse the Fenton reaction, in which H$_2$O$_2$ is converted to hydroxyl ion and hydroxyl radical (Equation 4). The combination of Equations 3 and 4 is the so-called Haber–Weiss reaction (Equation 5). When 'OH was produced, EDTA was added in addition to iron(III) ions to increase the effective concentration of iron, which is difficult to dissolve in water based buffers; iron complexed with EDTA is redox reactive. All reactions were started by adding xanthine oxidase, performed under constant stirring at 22°C and stopped by addition of 10 $\mu$g/ml superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6). In addition to ODPR, SP$_{1-11}$ was also exposed to reactive oxygen species H$_2$O$_2$ (1 and 0.1 mM) or HClO (added as calcium salt, 1 and 0.1 mM) The HClO reaction was stopped by L-methionine as scavenger, which at the end of the incubation was added to 10 mM final concentration.

(1) $\text{HX}^{+} \xrightarrow{\text{XAO}} \text{xanthine} \xrightarrow{\text{XAO}} \text{urate}$

(2) $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

(3) $Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$

(4) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$

(5) $H_2O_2 + O_2^- \rightarrow O_2 + OH^- + \cdot OH$

Samples from substance P exposed to $O_2^-$, 'OH, H$_2$O$_2$ or HClO were subjected to reverse phase high performance liquid chromatography (HPLC).

Results

Exposure to a continuous flux of superoxide caused formation of two major oxidation products, which had retention times similar to SP$_{1-11}$ (31.90 min) and SP$_{1-7}$ (27.68 min) and probably represent oxidative fragmentation of substance P$_{1-11}$. Synthetic soluble substance P (1 mg/ml) in RPMI-1640 (Gibco) was exposed to $O_2^-$ or hydroxyl radical ('OH) produced in a hypoxanthine/xanthine oxidase (EC 1.2.3.22) system in the presence of 1 mM deferoxamine (Desferal®, Ciba Pharmaceutical Co) and 40 mM $\alpha$-mannitol or 50 $\mu$M FeCl$_3$·6H$_2$O (Mallinckrodt, Paris, KY, USA) and 50 $\mu$M ethylenediaminetetraacetic acid (EDTA) (Sigma) respectively, as described in detail elsewhere. In the presence of these reagents, alterations observed are caused by $O_2^-$, although H$_2$O$_2$ is also formed in a so-called one-electron auto-oxidation or dismutation (Equation 2). Iron was added as iron(III) ions, so that the reaction would start immediately after addition of xanthine oxidase: in this case, iron(III) ions are first reduced to iron(II) ions (Equation 3). Iron(II) ions can then act as an electron donor and catalyse the Fenton reaction, in which H$_2$O$_2$ is converted to hydroxyl ion and hydroxyl radical (Equation 4). The combination of Equations 3 and 4 is the so-called Haber–Weiss reaction (Equation 5). When 'OH was produced, EDTA was added in addition to iron(III) ions to increase the effective concentration of iron, which is difficult to dissolve in water based buffers; iron complexed with EDTA is redox reactive. All reactions were started by adding xanthine oxidase, performed under constant stirring at 22°C and stopped by addition of 10 $\mu$g/ml superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6). In addition to ODPR, SP$_{1-11}$ was also exposed to reactive oxygen species H$_2$O$_2$ (1 and 0.1 mM) or HClO (added as calcium salt, 1 and 0.1 mM) The HClO reaction was stopped by L-methionine as scavenger, which at the end of the incubation was added to 10 mM final concentration.

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between the two phenylalanine residues at positions 7 and 8. Exposure to hydroxyl radicals produced similar fragments, but, in addition, two minor oxidation products with retention times 31.17 min and 18.33 min were produced (Figure 1). One of these had a retention time similar to SP3-11 (18.33 min) and although SP1-8 was not available for chromatographic analysis, these two minor hydroxyl radical oxidation products could, by deduction, represent an additional cleavage site between phenylalanine and glycine residues at positions 8 and 9.

If instead of ODFR, SP1-11 was exposed to reactive oxygen species H$_2$O$_2$ (1 and 0.1 mM) or HClO (added as calcium salt, 1 and 0.1 mM), fragmentation was not observed (only one reaction product was produced), but rather modification of the amino acid side chains and/or aggregation: H$_2$O$_2$ and HClO induced the formation of oxidation products with retention times 33.13 min and 32.04 min, respectively.

Oxidative modification of synthetic soluble substance P in aqueous solution was time-dependent. Fragmentation caused by superoxide and hydroxyl radical seemed to have a somewhat similar temporal profile (Figure 2). Oxidative modification induced by hydrogen peroxide was a relatively slow process (32.04 min product) compared to the almost immediate effect seen when
Table 1. Effect of PMA preactivated neutrophils on synthetic substance P. Human peripheral blood neutrophils (PMN) were isolated and preactivated by incubation in 50 μg/ml PMA at 37°C for 30 min. These preactivated cells were resuspended in 1 ml RPMI-1640 medium to which 1 mg/ml substance P was added. 100 μl aliquots were recovered at the times indicated and the cell free supernatant was analysed for substance P and its oxidation products using high performance liquid chromatography (HPLC) in peak area mode.

| Peakb | Incubation time with PMA preactivated neutrophils |
|-------|--------------------------------------------------|
|       | 1 min   | 5 min   | 15 min  | 30 min  | 1 h     | 2 h     |
| 31.60 min | 216    | 980    | 1882    | 1823    | 1472    | 1012    |
| 28.53 min | 263    | 554    | 204     | 1728    | 1916    | 2256    |
| 18.45   | 699    | 2641   | 7563    | 9753    | 10792   | 13165   |

*The areas of the peaks are expressed in arbitrary units calculated by the integrator (× 10⁻³).

bPeaks are defined here by their retention time in HPLC.

hypoiodous acid was used (33.13 min product) (Figure 3).

Synthetic SP₁₋₁₁ in aqueous solution was rapidly modified by PMA preactivated neutrophils. Peaks with retention times corresponding to those of SP₅₋₁₁, SP₁₋₇ and SP₉₋₁₁ appeared in the supernatant, whereas the peak corresponding to the minor SP₁₋₈ peak produced by 'OH in the HX/xanthine oxidase system was negligible (one representative experiment is shown in Table 1). In addition, peaks eluting at approximately 38.7 min, 40.2 min and 41.3 min were also found in the substance P–neutrophil supernatant aliquots (not shown).

**Discussion**

These results obtained using cell free conditions show that SP₁₋₁₁ can be fragmented by ODFR, both O₂⁻ and 'OH. Oxidative modifications of various free amino acids and proteins have received a lot of attention, but observations on the effect of ODFR on substance P have not been published. The effect of ODFR on various amino acids cannot be extrapolated to proteins or peptides due to their often complex secondary and tertiary structure, which will affect the sites accessible for the initial attack and the subsequent secondary effects such as intramolecular charge transfer reactions. Proteins, perhaps due to the usually relatively rigid structure possibly enforced by intra- and interchain disulphide bonds, are usually not fragmented by ODFR, although such an exposure may make them susceptible or mark them for a subsequent proteolytic attack. According to our finding, however, a small peptide, substance P, was fragmented by both O₂⁻ and 'OH. Substance P in aqueous phase acquires a mobile random coil configuration, with nonregular, nonrepeating dihedral angles of the peptide backbone with or without equilibrium between various random coil conformers. This may strain the peptide or α-carbon bonds and may contribute to their cleavage.

Some amino acids are more susceptible to oxidative damage than others. Phenylalanine containing an aromatic benzene ring is easily oxidized. Exposure of phenylalanine to 'OH will cause hydroxylation to the p-, m- and in particular e-position. Two such radicals can join together to give a dimer, that can lose water to form biphenyl. The close apposition of phenylalanine residues at positions 7 and 8 in a flexible random coil SP₁₋₁₁ might favour the formation of such biphenyls, which could cause a sudden change, a nick, in the stereoconfiguration. That non-enzymatic, conformational changes can cleave even peptide bonds is suggested by the action of catalytic antibodies. An antibody to vasoactive intestinal peptide (VIP) can cause cleavage of a peptide bond in full-length VIP₁₋₁₈. Interestingly, against expectations, the scissile bond was not part of the antigenic determinant involved in the high affinity antigen–antibody binding. Therefore, the relatively strong interactive forces involved in binding of the catalytic antibody to VIP₁₋₁₈ are unlikely to be directly responsible for the peptide bond hydrolysis. Competitive binding studies using various synthetic VIP peptide fragments were used to map the antibody binding epitope to amino acid residues 22-28. This binding was able to cause a cleavage at a distant scissile Glu¹⁶-Met¹⁷ bond, perhaps via conformational changes induced by binding of the catalytic antibody.

Neuropeptide peptide bonds are cleaved by various well described exo- and endopeptidases. Actually, ODFR usually lead to formation of α-carbon centred radicals and, in the presence of oxygen, peroxyl radicals, which decompose to fragment the polypeptide chain at the α-carbon rather than at peptide bond. Accordingly, the cleavage sites observed in the present study, i.e. between the phenylalanine residues at positions 7 and 8 and, with 'OH also between the phenylalanine and glycine at positions 8 and 9, may be located at the α-carbon rather than the peptide bond. Obviously, the hypothesis about the fragments of substance P generated by oxygen radical exposure has to be validated in future studies with appropriate nuclear magnetic resonance and mass spectroscopy studies allowing the identification of the structure of the generated metabolites.

In contrast to O₂⁻ and 'OH, H₂O₂ is, by definition, not a radical; it does not contain unpaired electrons i.e. electrons occupying an atomic or molecular orbital by itself. It is not particularly reactive either, compared to ODFR. On the other hand, H₂O₂, like water, as a small, uncharged polar molecule, has a very high...
permeability coefficient compared to e.g. charged O_2^- . It can therefore, pass through lipid bio-
membranes. It can also, according to Equation 4 (see above), lead to the formation of the highly reactive hydroxyl radical in the presence of transition metal ions like iron and copper. In the present study, when H_2O_2 was added to an aqueous solution, it did not cause fragmentation of SP_{1-11}, but instead a slowly progressive structural modification, which was reflected in altered hydrophobicity of the reaction product in reverse phase HPLC. In contrast, HClO caused a rapid modification of SP_{1-11}, which again was reflected in altered hydrophobicity. It was noteworthy that the present study, when H_2O_2 was added to an aqueous solution, it did not cause fragmentation of SP_{1-11}, but instead a slowly progressive structural modification, which was reflected in altered hydrophobicity of the reaction product in reverse phase HPLC. In contrast, HClO caused a rapid modification of SP_{1-11}, which again was reflected in altered hydrophobicity. It was noteworthy that the HClO-induced product had a different retention time than the H_2O_2-induced oxidation product. Although not studied in structural detail, it is likely that HClO caused conversion of the methionine residue at position 11 to a corresponding methionine sulphoxide (MetSO) or perhaps even to a methionine sulphone (MetSO_2). Also this reaction pathway would seem to be of potential relevance in vivo in inflammation, because under such circumstances HClO is formed from H_2O_2 by myeloperoxidase in the presence of chloride ion. Chloride ion is one of the most common ions in the extracellular tissue fluid and myeloperoxidase is stored in and, upon activation, released from the primary or azurophil granules of the PMN. PMA is a direct activator of the protein kinase C and will, by phosphorylation to serine and threonine residues of some as yet mostly unknown target proteins, activate the cell membrane NADPH oxidase to produce O_2^- . This will spontaneously dismutate to H_2O_2 at a rate of 2 \times 10^{-5} \text{ M s}^{-1}. In the presence of trace amounts of iron, always present in 'average' reaction mixtures without added metal, usually at about 1 \muM as assessed by atomic absorption analysis, hydroxyl radicals also are likely to be formed. The interpretation of PMA preactivated neutrophil experiments is complicated by cellular uptake and by simultaneous activation of neutrophil-mediated exocytosis as was suggested by the presence of peaks other than those produced in the cell free HX/xanthine oxidase system. Neutrophils contain in their primary granules cathepsin G, which, according to the bond specificity is able to cleave substance P. In addition, neutrophils contain an integral membrane protein known as neutral endopeptidase EC 3.4.24.11, also known as the common acute lymphoblastic leukaemia associated antigen CALLA and as enkephalinase, which is also able to cleave substance P. Therefore, even if untreated neutrophils would cause substance P degradation or superoxide scavenger superoxide dismutase would not be able to inhibit substance P degradation, it is not possible to exclude the role of oxidative degradation because there are alternative and complementary degradative pathways. However, these results suggest that substance P exposed to PMA preactivated neutrophils is modified as if exposed to superoxide or hydroxyl radicals in a cell free HX/xanthine oxidase system in vitro.

It therefore seems that various ODFR and reactive oxygen species can cause oxidant specific and time-dependent modification of synthetic SP_{1-11} in aqueous phase by inducing fragmentation, modification of side chains and aggregation. According to the present findings, it seems likely that oxidative modification also has to be taken into consideration when the paracrine actions of SP_{1-11} in inflammatory diseases and processes are regarded: the distance from the axon terminal to the potential site of action may at the paracrine site of action be more than 10,000 longer than in the more concealed synaptic spaces.

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