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Research paper

Formation of 2-nitrophenol from salicylaldehyde as a suitable test for low peroxynitrite fluxes

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ABSTRACT

There has been some dispute regarding reaction products formed at physiological peroxynitrite fluxes in the nanomolar range with phenolic molecules, when used to predict the behavior of protein-bound aromatic amino acids like tyrosine. Previous data showed that at nanomolar fluxes of peroxynitrite, nitration of these phenolic compounds was outcompeted by dimerization (e.g. biphenols or dityrosine). Using 3-morpholino sydnonimine (Sin-1), we created low fluxes of peroxynitrite in our reaction set-up to demonstrate that salicylaldehyde displays unique features in the detection of physiological fluxes of peroxynitrite, yielding detectable nitration but only minor dimerization products.

By means of HPLC analysis and detection at 380 nm we could identify the expected nitration products 3- and 5-nitrosalicylaldehyde, but also novel nitrated products. Using mass spectrometry, we also identified 2-nitrophenol and a not fully characterized nitrated dimerization product. The formation of 2-nitrophenol could proceed either by primary generation of a phenoxy radical, followed by addition of the NO_2-radical to the various resonance structures, or by addition of the peroxynitrite anion to the polarized carbonyl group with subsequent fragmentation of the adduct (as seen with carbon dioxide). Interestingly, we observed almost no 3- and 5-nitrosalicylic acid products and only minor dimerization reaction.

Our results disagree with the previous general assumption that nitration of low molecular weight phenolic compounds is always outcompeted by dimerization at nanomolar peroxynitrite fluxes and highlight unique features of salicylaldehyde as a probe for physiological concentrations of peroxynitrite.

1. Introduction

Oxidative stress conditions are a hallmark of a large number of cardiovascular, neurodegenerative and inflammatory diseases as well as cancer [1–6]. Recent evidence, that is mainly based on genetic animal models with altered formation and detoxification of reactive oxygen species (ROS), suggests that oxidative stress is not only a consequence, but also a trigger of these pathological states (summarized in [7]). Among the various forms of ROS in biological systems, peroxynitrite, the product of the fast combination of the nitric oxide radical ($^{\bullet}NO$) and superoxide anion radical ($O_2^{\bullet -}$) [8], is of special interest [9]. Since $^{\bullet}NO$ has important functions as a messenger, its trapping by $O_2^{\bullet -}$ becomes a regulatory process and even the resulting peroxynitrite possesses new functions in redox regulation [10,11].

The assessment of peroxynitrite formation under physiological conditions is a challenge since the low fluxes of $^{\bullet}NO$ and $O_2^{\bullet -}$ are in the nano- to micro-molar range and its high reactivity only allows indirect measurements of its reaction products with target molecules in vivo [12]. Fortunately, the nitration of protein-bound tyrosine residues can serve as a footprint [13] that can be detected in a large number of the afore mentioned diseases [1,14,15]. In case of manganese superoxide dismutase [16,17] or prostacyclin synthase [18,19] such tyrosine nitrations can lead to inactivation, in other cases like α-synuclein to conformational changes [20,21] and possible pathological consequences have been suggested.

A valid approach to determine peroxynitrite in biological systems is the addition of low molecular weight compounds that, by a fast reaction with peroxynitrite, outcompete biological targets and

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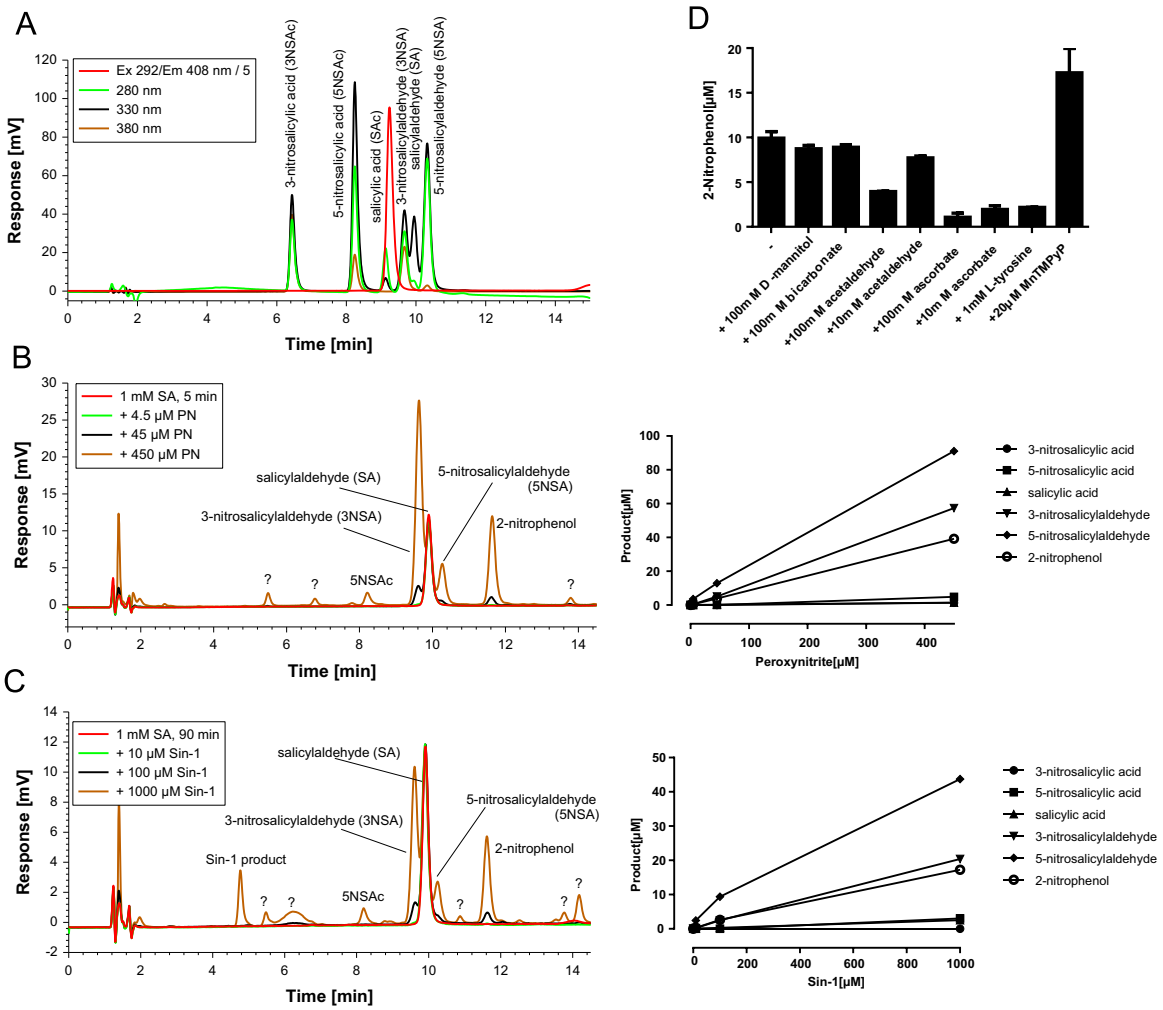


Fig. 1. (A) HPLC detection of external standards for salicylaldehyde and its expected nitration and oxidation products. All compounds were detected by their absorption at 280, 330 and 380 nm, as well as by their fluorescence (Ex. 292 nm/Em. 408 nm). Original chromatograms are shown. (B) HPLC detection of the reaction products of authentic peroxynitrite with salicylaldehyde. Samples of varied peroxynitrite concentration were incubated with 1 mM salicylaldehyde for 5 min at 37 °C and subjected to HPLC measurements with detection at 380 nm absorbance wavelength. Original chromatograms as well as quantification of products are shown. (C) HPLC detection of the reaction products of the peroxynitrite donor Sin-1 with salicylaldehyde. Samples of varied Sin-1 concentration were incubated with 1 mM salicylaldehyde for 90 min at 37 °C and subjected to HPLC measurements with detection at 380 nm absorbance wavelength. Original chromatograms as well as quantification of products are shown. (D) Peroxynitrite (150 μM) was incubated with 1 mM salicylaldehyde for 5 min at 37 °C in the presence of the hydroxyl radical scavenger D-mannitol, the carbon dioxide donor $KHCO_3$, the competitive reaction target acetaldehyde, or the 1e-antioxidants/free radical scavengers ascorbate and L-tyrosine. Before the reaction all stock solutions (in 0.2 M potassium phosphate buffer at pH 7.4) were checked for pH and adjusted when necessary to 7.4 (this was the case for the stock solution of 100 mM ascorbic acid and 100 mM bicarbonate). The samples were subjected to HPLC measurements with detection at 380 nm absorbance wavelength. Data are mean ± SEM of 2 independent experiments.

form stable and characteristic products. Evidently, tyrosine in its free form can be used since it forms 3-nitrotyrosine upon reaction with peroxynitrite [22] as also observed with phenol [23]. However, at physiological fluxes of peroxynitrite mainly dimerization products are formed with tyrosine [24] and phenol [23] and even doubts have been raised that $^{\bullet}NO$ and $O_2^{\bullet -}$ can form 3-nitrotyrosine [25–27]. Subsequent studies have clearly shown that $^{\bullet}NO$ and $O_2^{\bullet -}$ can form 3-nitrotyrosine, especially when reacting with protein-bound tyrosine [22,28–30] and also biological proof for this reaction was provided using activated immune cells [31,32]. A detailed chemical view on the relevant reaction steps in the tyrosine/$^{\bullet}NO/O_2^{\bullet -}$ system was later provided by Goldstein and coworkers [33]. In addition, 3-nitrotyrosine may also result from the enzymatic activation of nitrite by peroxidases and hydrogen peroxide. Depending on the nature of invading pathogens, nitration of tyrosine residues will either involve peroxynitrite or the peroxidase/nitrite/hydrogen peroxide [34].

So far there is no literature available on the reaction of salicylaldehyde with peroxynitrite. Here, we show that salicylaldehyde is nitrated efficiently by nanomolar fluxes of nitric oxide/superoxide, generated by the peroxynitrite donor 3-morpholino sydnonimine (Sin-1), at a level comparable to bolus additions of authentic peroxynitrite. In contrast, the NO donor spermine NONOate showed almost no reactivity and peroxidase-driven nitration of salicylaldehyde only occurs at high nitrite and hydrogen peroxide concentrations.

2. Materials and methods

2.1. Chemicals

Sin-1 hydrochloride (3-morpholinosydnonimine HCl) was from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). Spermine NONOate (N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine) was purchased from Cayman Chemical Company (Ann Arbor, USA). Xanthine oxidase (E.C. 1.1.3.22) grade III from buttermilk was purchased from Sigma

(Steinheim, Germany). Salicylaldehyde and all related products as well as all buffer salts were purchased from Sigma-Aldrich, Fluka or Merck at the highest purity grade available. An alkaline peroxynitrite stock solution (80 mM in aqueous NaOH) was synthesized according to the "quenched flow" method as previously published [35] and stored at −80 °C.

2.2. Detection and quantification of salicylaldehyde products

Reactions were performed in 0.1 M potassium phosphate buffer at pH 7.4 and 37 °C and all reactive compounds were handled as described previously [30]. Authentic peroxynitrite was rapidly mixed using a Vortex mixer with salicylaldehyde and incubated for 5 min. All reactions of Sin-1, spermine NONOate, NADPH oxidase inhibitor-VAS2870, xanthine oxidase with hypoxanthine as well as horseradish peroxidase/nitrite/hydrogen peroxide were incubated for 90 min under the above described conditions. 50 μl aliquots of the samples were subjected to high performance liquid chromatography (HPLC) analysis. The system consisted of a control unit, two pumps, a mixer, detectors, a column oven, a degasser, an autosampler (AS-2057 plus) from Jasco (Groß-Umstadt, Germany), and a C18-Nucleosil 100-3 (125 × 4) column from Macherey & Nagel (Düren, Germany). A high-pressure gradient was employed with the organic solvent (90 vv% acetonitrile/10 vv% water) and 50 mM citrate buffer pH 2.2 as mobile phases with the following percentages of the organic solvent: 0 min, 10%; 14.5 min, 42%; 15 min, 10%; 16.5 min, 10%. The flow was 1 ml/min, compounds were detected by their absorption at 280, 330 and 380 nm, and salicylic acid was also detected by fluorescence (Ex. 292 nm/Em. 408 nm). Typical retention times of all standards are shown in Fig. 1.

2.3. Characterization of new products by high resolution mass spectrometry

LC–MS experiments were carried out on an Agilent 1290 Infinity UHPLC system (Agilent Technologies, Santa-Clara, California, U.S.A.) equipped with a binary pump and an autosampler. The chromatographic separation was performed using an Acquity UPLC BEH C18 1.7 μm (2.1 × 50 mm^2) column (Waters, Eschborn, Germany). A gradient was applied using a mobile phase 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.3 ml/min with the following percentages of (B): 0 min, 2%; 1 min, 2%; 5 min, 95%; 5.5 min, 95%: 6 min, 2%. The LC system was coupled to an Agilent 6540 Accurate-Mass Q-TOF mass spectrometer equipped with atmospheric pressure chemical ionization (APCI) source. APCI was performed in negative ion mode scanning m/z from 100 to 500 amu at a scan rate of 5 spectra s^{-1},using the following source settings: gas temperature 360 °C, gas flow 8 l/min, nebulizer 35 psi, capillary voltage 3200 V, fragmentator: 120 V. Collision energy in MS/MS mode was 12 V. Data were acquired and analyzed with Masshunter Workstation Software (Agilent Technologies).

2.4. Isolation of white blood cells

Leukocytes and the sub-fraction of neutrophils were isolated from human whole blood using the dextran sedimentation and Ficoll centrifugation protocol as described previously [36]. Total blood cell count and the purity of the fractions were evaluated using an automated approach with a hematology analyzer KX-21N (Sysmex Europe GmbH, Norderstedt, Germany). The typical constitution of the blood cell fractions was previously described in detail [37].

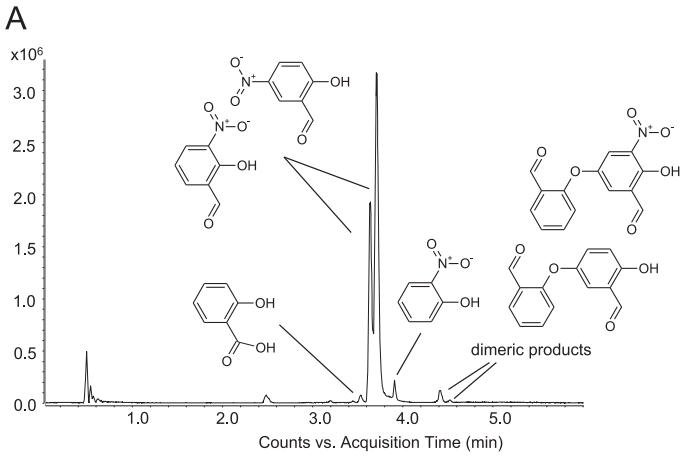
3. Results

3.1. Reaction of salicylaldehyde with authentic peroxynitrite

In order to determine the effectiveness of salicylaldehyde as a detection molecule for the presence of peroxynitrite in the experimental system, we started our investigations by utilizing an exploratory set-up with excess quantities of authentic peroxynitrite as bolus additions. By means of HPLC we were able to detect and quantify almost all reaction products, among them being the expected 3- and 5-nitrosalicylaldehyde at high yield, 5-nitrosalicylic acid at low yield and a prominent unknown nitration product, which was later identified as 2-nitrophenol (Fig. 1A and B). There was an almost linear increase in these products with increasing peroxynitrite concentrations. Interestingly, there were only traces of salicylic acid and no 3-nitrosalicylic acid formed under these conditions (Fig. 1B). Formation of 2-nitrophenol by 150 μM peroxynitrite was suppressed by approximately 20% and 46% by 50 or 250 μM uric acid, respectively, whereas the yield of nitration was decreased by 37% and 64% by 50 or 250 μM selenomethionine, respectively (not shown). Both inhibitors have been used since uric acid efficiently scavenges nitrogen dioxide formed by homolytic cleavage of peroxynitrite [30,38] and seleno-methionine readily accepts an oxygen atom from peroxynitrite anion [30,39]. With phenol as a substrate, uric acid had been found to be a more efficient inhibitor for the nitration than seleno-methionine (IC_{50} 40 versus 250 μM) [30]. The less efficient inhibition of the reaction of salicylaldehyde and peroxynitrite by both compounds might reflect fast reaction kinetics but does not provide direct evidence for a polar or radical-based mechanism of the reaction. For the purpose of our search for a selective peroxynitrite indicator, the exact mechanism is not important. Nevertheless, additional experiments with antioxidant compounds were performed for better insight into the reaction mechanism. D-mannitol (hydroxyl radical scavenger), bicarbonate (carbon dioxide source), acetaldehyde (competitive reaction target), ascorbate and L-tyrosine (1e-antioxidants), MnTMPyP (manganese porphyrin) were used at high concentrations (Fig. 1D). D-mannitol had no effect excluding an essential role of free hydroxyl radicals for the product yield. Bicarbonate had no effect on the nitration yield speaking against an essential role of peroxynitrite–carbon dioxide adduct formation for the nitration mechanism. Acetaldehyde showed competitive inhibition of salicylaldehyde nitration at 100-fold excess, whereas at 10-fold excess the inhibitory effect was only minor. Ascorbate and L-tyrosine were quite efficient supressors of salicylaldehyde nitration when incubated at equimolar or 10-fold excess over salicylaldehyde, supporting at least radical intermediates in the reaction mechanism of salicylaldehyde and peroxynitrite. Finally, the manganese porphyrin increased the nitration yield, again supporting radical intermediates that could interact with transition metals.

3.2. Reaction of salicylaldehyde with nitric oxide/superoxide generated by Sin-1

In order to determine the effectiveness of salicylaldehyde as a detection molecule for the presence of nanomolar (physiological) fluxes of peroxynitrite, we incubated the salicylaldehyde solution with the peroxynitrite donor Sin-1. Especially at lower Sin-1 concentrations (≤ 100 μM), the peroxynitrite donor was almost as efficient as authentic peroxynitrite bolus additions in nitrating salicylaldehyde (Fig. 1C). Besides high amounts of 3- and 5-nitrosalicylaldehyde as well as 2-nitrophenol, low concentrations of 5-nitrosalicylic acid as well as some other products with longer retention times were formed (which would be compatible with the formation of low levels of nitrated dimerization products).



Retention time [min]	m/z observed	molecular formula	m/z theoretical	deviation in ppm
3.53	137.02447	C7H6O3	137.02442	0.4
3.64	166.01459	C7H5N1O4	166.01458	0.1
3.72	121.02953	C7H6O2	121.0295	0.2
3.72	166.01471	C7H5N1O4	166.01458	0.8
3.91	138.01946	C6H5N1O3	138.01967	1.5
4.38	286.03471	C14H9N1O6	286.0.3571	3.5
4.41	241.05000	C14H10O4	241.05063	2.6
4.52	241.04996	C14H10O4	241.05063	2.8

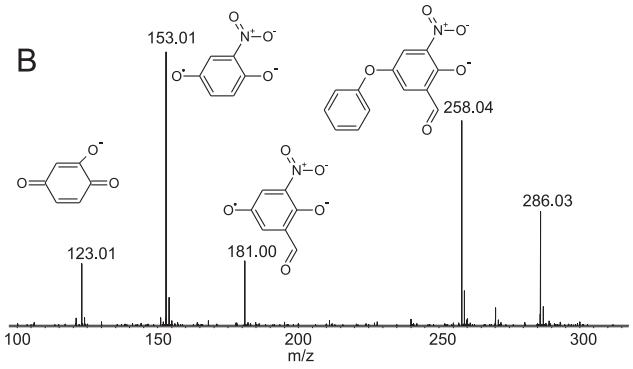


Fig. 2. LC–MS and LC–MS/MS analysis of reaction products of Sin-1 with salicylaldehyde. (A) LC–MS base peak chromatogram (APCI negative ion mode) with suggested structures based on accurate mass determination. The table shows characteristics of all detected products: Retention time, observed mass/charge ratios (*m*/*z*), molecular formulas, calculated mass/charge ratios (m/z) of $[M–H]^-$-ions and measurement errors (ppm). (B) MS/MS spectrum of dimeric reaction product at 3.38 min (*m*/*z* 286) with suggested fragment ions.

Formation of 2-nitrophenol by 500 μM Sin-1 was suppressed by approximately 42% to 55% by 100 or 300 U/ml polyethylene-glycolated superoxide dismutase, respectively, whereas 2-nitrophenol formation was decreased by only 12% by 100 μM uric acid and by only 19% by 100 μM seleno-methionine, respectively (not shown). These inhibitor data demonstrate that removal of superoxide from the system prevents the nitration of salicylaldehyde but cannot identify the exact nature, polar or free radical-based, of the reaction mechanism [30].

3.3. Identification of new products

The unknown products from reactions shown in Figs. 2 and 3 were identified by liquid chromatography and high resolution mass spectrometry. High resolution accurate mass measurements can be used to determine the elemental composition of an unknown compound due to its characteristic mass defect. Fig. 2 shows a typical base peak chromatogram of the salicylaldehyde and Sin-1 reaction with suggested structures fitting to the major peaks as well as a table summarizing the accurate mass determinations and molecular formulas of all detected products. Assuming C, H, O, N compositions and measurement error <4 ppm, unambiguous identifications of molecular formulas for all reaction products were obtained. The unknown nitration product in Fig. 1B and C ($t_R=11.6$ min) was identified as 2-nitrophenol, which was later confirmed and quantified by external and internal standards. The unknown nitration product in Fig. 1C ($t_R=14.2$ min) was identified as a dimerization product of salicylaldehyde and 3-nitrosalicylaldehyde, which could not be quantified due to lack of suitable commercial standard. The dimeric structure was supported by MS/MS-Data. Fig. 2 shows the APCI negative ion MS/MS spectrum of m/z 286 with suggested fragment ions.

3.4. Reaction of salicylaldehyde with nitric oxide generated by spermine NONOate (SPE/NO)

In order to verify the effectiveness of salicylaldehyde as a detection molecule in the presence of nanomolar (physiological) fluxes of nitric oxide alone, we incubated the aerobic salicylaldehyde solution with the nitric oxide donor SPE/NO. Importantly, the nitric oxide donor alone up to a concentration of 1000 μM yielded only marginal nitration of the salicylaldehyde (Fig. 3A), which was only 1/30 of the nitration observed with the peroxynitrite donor Sin-1. This was an important finding since usually phenolic compounds are nitrated by higher SPE/NO concentrations at atmospheric oxygen tensions in the buffer solutions (most probably by an autoxidation mechanism of $^{\bullet}$NO with molecular oxygen yielding nitrogen dioxide radicals [40]).

3.5. Reaction of salicylaldehyde with superoxide generated by xanthine oxidase (XO).

Since peroxynitrite is formed from $^{\bullet}$NO and superoxide, it was important to also identify salicylaldehyde products upon reaction with superoxide to exclude any potential interference with the product pattern of peroxynitrite (although nitration by superoxide can be excluded). The only detectable product from the reaction solution of salicylaldehyde and xanthine oxidase/hypoxanthine was salicylic acid, which increased in a linear fashion in dependence of the XO concentration (Fig. 3B). Although these data implied efficient oxidation of salicylaldehyde by superoxide or hydrogen peroxide, subsequent experiments with XO (20 mU/ml)

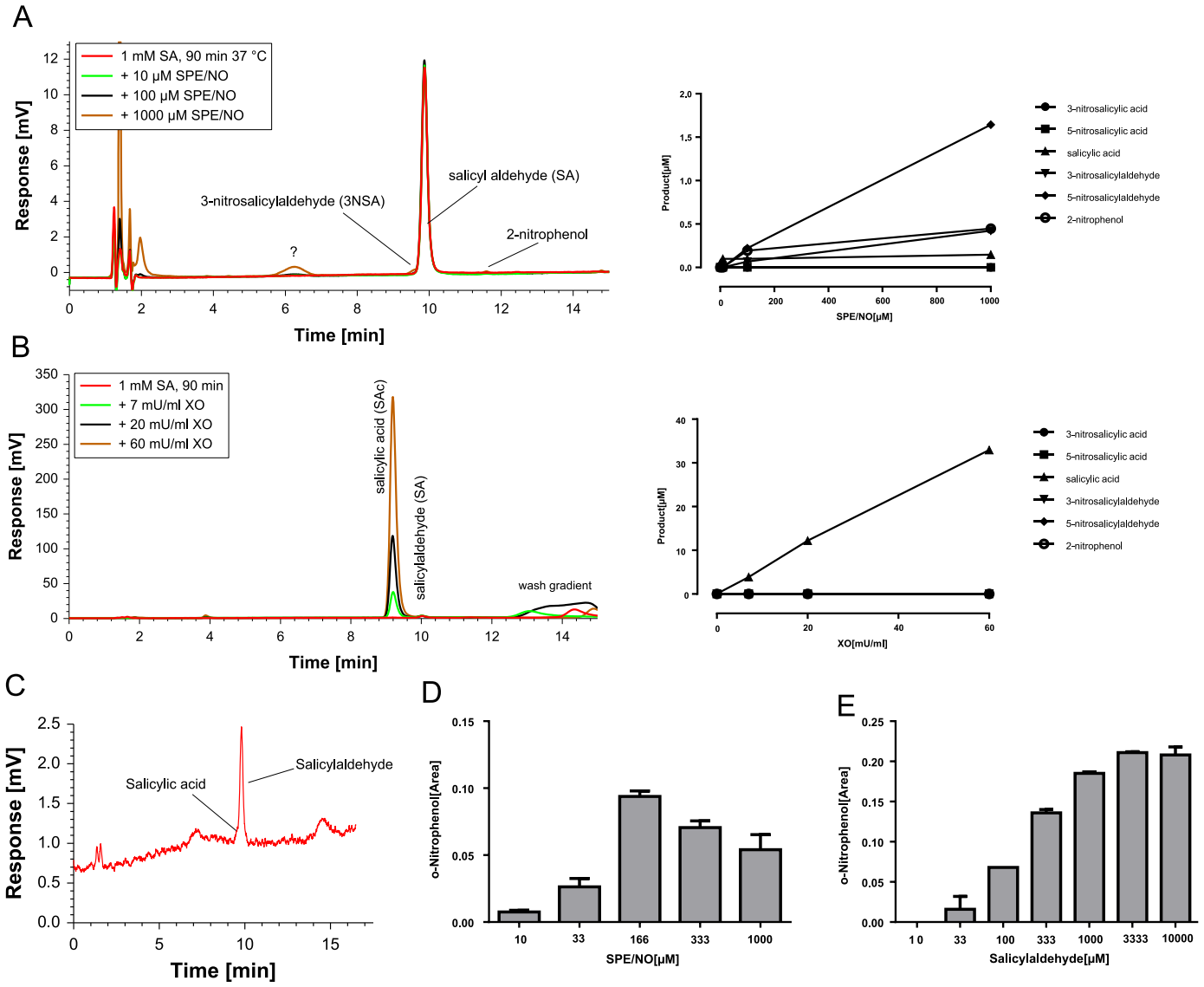


Fig. 3. (A) HPLC detection of the reaction products of the nitric oxide donor spermine NONOate (SPE/NO) with salicylaldehyde. Samples of varied SPE/NO concentration were incubated with 1 mM salicylaldehyde for 90 min at 37 °C and subjected to HPLC measurements with detection at 380 nm absorbance wavelength. Original chromatograms as well as quantification of products are shown. (B) HPLC detection of the reaction products of the superoxide producing system xanthine oxidase (XO) with salicylaldehyde. Samples of varied XO concentration were incubated with 1 mM hypoxanthine and 1 mM salicylaldehyde for 90 min at 37 °C and subjected to HPLC measurements with fluorescence detection (Ex. 292 nm/Em. 408 nm wavelengths). Original chromatograms as well as quantification of products are shown. (C) Representative reaction mixture of 1 mM hydrogen peroxide with 1 mM salicylaldehyde for 90 min at 37 °C and subjected to HPLC measurements with fluorescence detection (Ex. 292 nm/Em. 408 nm wavelengths). (D) Reaction of XO (33.3 mU/ml) with increasing concentrations of SPE/NO and 1 mM salicylaldehyde and 1 mM hypoxanthine for 90 min at 37 °C and pH 7.4. Data are mean ± SEM of 4 independent experiments. (F) Reaction of Sin-1 (100 µM) with increasing concentrations of salicylaldehyde for 90 min at 37 °C and pH 7.4. Data are mean ± SEM of 2 independent experiments.

and hypoxanthine (1 mM) in the presence of 100 or 300 U/ml polyethylene-glycolated superoxide dismutase did not decrease the yield of salicylic acid (even a slight increase was observed at higher SOD concentration) and salicylaldehyde oxidation was also present without the XO substrate hypoxanthine (not shown). Apparently, the oxidation of salicylaldehyde by XO and hypoxanthine is a result of the direct conversion of the aldehyde by the oxidoreductase activity.

By performing another control with hydrogen peroxide, we conclude that salicylaldehyde does not yield salicylic acid from salicylaldehyde (Fig. 3C), indicating that hydrogen peroxide as a break-down product from xanthine oxidase reaction is unlikely to cause the oxidation of the aldehyde. Reaction mixtures with a fixed concentration of XO (33.3 mU/ml) and increasing concentrations of SPE/NO yielded a maximum of 2-nitrophenol formation, clearly identifying peroxynitrite as the nitrating species (Fig. 3D). The optimal concentration of salicylaldehyde for the nitration yield was already reached at 1 mM and showed a plateau for product formation even when the concentration of salicylaldehyde was further increased (Fig. 3F).

3.6. Biological utilization of salicylaldehyde as a detection molecule

One of the important parameters for the probe is its suitability for measurements in biological samples. We are showing here that salicylaldehyde can be used for the detection of low fluxes of peroxynitrite in a cellular system consisting of isolated human neutrophils (PMN) upon stimulation with zymosan A (Zym A) and simultaneous incubation with the •NO donor SPE/NO. Under these conditions the formation of 2-nitrophenol from salicylaldehyde was observed (Fig. 4A). Although the yield of nitrated product was very low, these data demonstrate that salicylaldehyde could be a

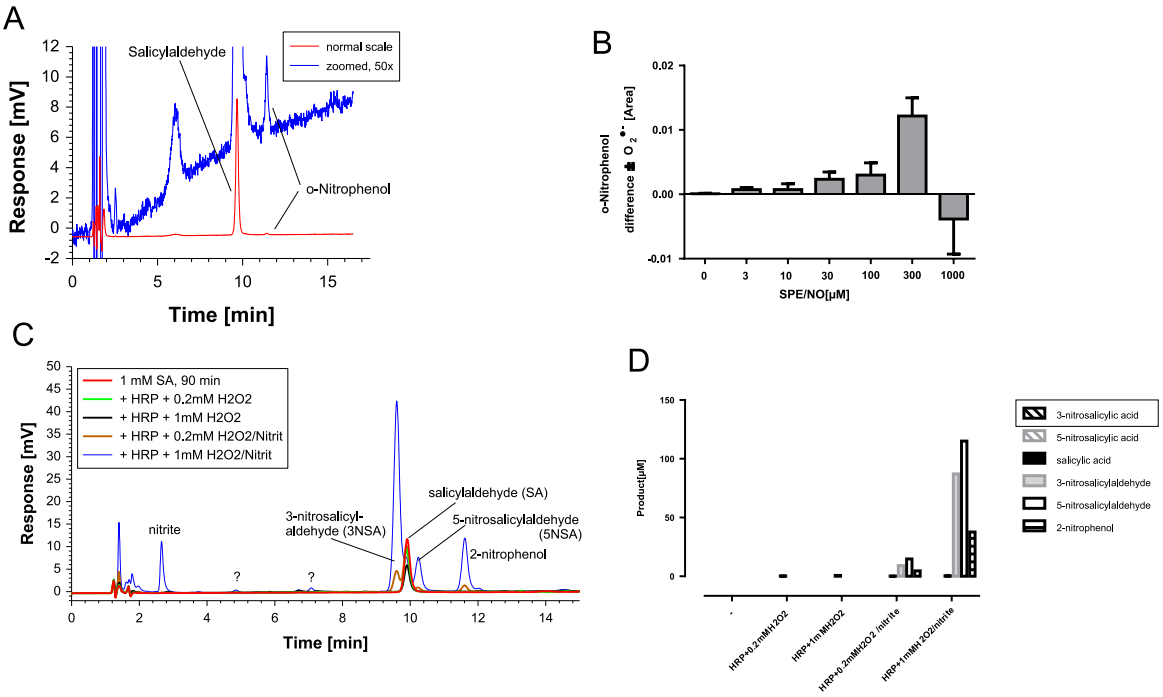


Fig. 4. HPLC detection of the reaction products of hydrogen peroxide or isolated human neutrophils with salicylaldehyde. (A) Samples of human neutrophils (10^6 cells/ml) stimulated with phorbol ester (1 μM) were incubated with 1 mM salicylaldehyde in the presence of spermine NONOate (100 μM) for 90 min at 37 °C and subjected to HPLC measurements with detection at 380 nm absorbance wavelength. Original chromatograms are shown. (B) Samples of human neutrophils (2×10^6 cells/ml) stimulated with zymosan A (50 μg/ml) were incubated with 1 mM salicylaldehyde in the presence of increasing spermine NONOate concentrations for 90 min at 37 °C in the presence or absence of the NADPH oxidase inhibitor VAS2870 (10 μM). The quantification shows the difference between 2-nitrophenol yields without minus with VAS2870 treatment. (C, D) HPLC detection of the reaction products of horseradish peroxidase (HRP), nitrite and hydrogen peroxide with salicylaldehyde. Samples of fixed HRP (0.1 μM) and varied nitrite/hydrogen peroxide concentrations were incubated with 1 mM salicylaldehyde for 90 min at 37 °C and subjected to HPLC measurements with detection at 380 nm absorbance wavelength. Original chromatograms (C) as well as quantification of products (D) are shown.

potential probe for the detection of peroxynitrite under cellular conditions. Increasing concentrations of SPE/NO in the presence of stimulated neutrophils resulted in a maximum of 2-nitrophenol when the background (SPE/NO alone effect) was subtracted (Fig. 4B). This was done by calculating the 2-nitrophenol difference between NADPH oxidase (oxidative burst)-stimulated and NADPH oxidase blocked (VAS2870) neutrophils. The VAS2870-treated cells lack substantial superoxide formation [41,42] and accordingly only the increasing SPE/NO concentrations will contribute to the overall 2-nitrophenol formation. In contrast, in the absence of VAS2870, the oxidative burst-generated superoxide will react with SPE/NO to produce peroxynitrite, which again should yield a maximum at approximately equimolar concentrations of superoxide and NO.

3.7. Interference of salicylaldehyde peroxynitrite detection with peroxidase-catalyzed nitration in the presence of inorganic nitrite and hydrogen peroxide

Previous work reported a significant role of peroxidase-triggered nitration reactions in the presence of nitrite/hydrogen peroxide [25,26], although this mechanism is not operative in all inflammatory conditions (mainly depending on the pathogen) [34]. It is known that horseradish peroxidase (HRP) in the presence of hydrogen peroxide and nitrite may lead to similar product formation as the peroxynitrite reaction mixture and even may involve peroxynitrite as an intermediate [43]. Indeed, we observed appreciable nitration levels of salicylaldehyde at high nitrite/hydrogen peroxide concentrations (both 1 mM) (Fig. 4C and D), whereas the 200 μM mixture yielded less nitration than 100 μM Sin-1. Based on these findings a contribution of peroxidase-catalyzed nitration of salicylaldehyde in biological samples cannot be excluded on a mechanistic basis.

4. Discussion

One of the major challenges in the field of reactive oxygen species is the lack of specific tools to detect and quantify reactive oxygen and nitrogen species [44]. Measurement of such molecules in biological systems constitute even bigger problems [45]. In our experimental set-up we selected salicylaldehyde as a potential marker for peroxynitrite. The peroxynitrite molecule possesses an extremely short half-life and is highly reactive either with other free radicals present in the system, or with surrounding oxidizable biological molecules (e.g. reactive thiol, thioethers, amino or aldehyde groups, as well as activated aromatic rings such as phenol) [46]. In our approach, we selected salicylaldehyde as a probe since in addition of being a nitratable phenol it contains an oxidizable aldehyde group. Together, both groups could provide selectivity in the action of peroxynitrite on this compound. In order to validate our concept that salicylaldehyde can be used as a selective peroxynitrite detector, we used different chemical systems to generate peroxynitrite or its precursor radicals, superoxide and nitric oxide. As shown in Fig. 1A, salicylaldehyde was able to react with synthesized $ONOO^-$ in a dose dependent manner and gave characteristic nitrated products. The HPLC profile clearly depicts the presence of 3- and 5-nitrosalicylaldehyde, as it is confirmed by the HPLC analysis of the appropriate standards (Fig. 1B). At the same time almost no dimerization products were identified by this method. While conducting the initial measurements, we observed several products, for which appropriate standards were lacking. In order to characterize them, we performed LC/MS with MS/MS analysis and were able to show that the major unidentified product was 2-nitrophenol. In minor quantities we observed novel dimerization products, as can be taken from Figs. 1C and 2.

Hence, with bolus additions of chemically synthesized peroxynitrite the expected nitration in the ortho- and para-positions of

the phenolic group as well as some radical-derived dimerization products could be verified. Of main interest was the identification of 2-nitrophenol as a major metabolite. The elimination of the formyl group with concomitant introduction of the nitro-group appears like a special feature of peroxynitrite. For the purpose of our investigation it is of minor significance whether the formyl group leaves as a formiate, as the sum equation suggests, or whether the mechanism involves primary formation of a phenoxy radical with secondary addition of the nitrogen dioxide radical to the radical resonance structures in ortho- and para-positions of the phenolic group (although we could detect no para-product in our reactions) or whether the peroxynitrite anion adds to the polarized carbonyl group with secondary radical fragmentation of the peroxide intermediate. It rather was the question whether 2-nitrophenol formation is characteristic for peroxynitrite as a biological intermediate.

With this respect it was essential to show that the simultaneous generation of $^{\bullet}NO$ and $O_2^{\bullet-}$ as the bioloogical source of peroxynitrite gave the same results as with authentic peroxynitrite. The autoxidation of Sin-1 provides equal fluxes of $^{\bullet}NO$ and $O_2^{\bullet-}$, lead to formation of 2-nitrophenol and addition of PEG–SOD inhibited this nitration reaction (Fig. 1C). The control with $^{\bullet}NO$ generated by SPE/NO alone was negative at low concentrations of the $^{\bullet}NO$ donor but resulted in some 2-nitrophenol formation at high SPE/NO concentrations which was also observed previously [22], probably through autoxidation of $^{\bullet}NO$ by molecular oxygen leading to nitrogen dioxide radicals (Fig. 3A). However, such high fluxes of $^{\bullet}NO$ are beyond the physiological range as well as the oxygen concentrations in tissues. The formation of nitrogen dioxide radicals from $^{\bullet}NO$ and O_2 proceeds with 3rd order kinetics and therefore is negligible under cellular levels of both reactants. Controls with superoxide generated by xanthine oxidase only resulted in oxidation of salicylaldehyde to salicylic acid, an obviously XO dependent direct oxidation (Fig. 3B). Importantly, simultaneous generation of $^{\bullet}NO$ and $O_2^{\bullet-}$ by SPE/NO and XO with increasing concentrations of the $^{\bullet}NO$ donor (thereby shifting the $^{\bullet}NO$ / $O_2^{\bullet-}$ ratio) resulted in a bell-shaped curve as previously reported for peroxynitrite-specific detection probes [22,40,47], clearly indicating specific reaction of salicylaldehyde with peroxynitrite to form 2-nitrophenol (Fig. 3C).

On the other hand, if superoxide is generated under cellular conditions in our set-up with phorbol ester- or zymosan A-stimulated human neutrophils and NO was co-generated from the NO donor SPE/NO, added salicylaldehyde yielded 2-nitrophenol in small but significant amounts (Fig. 4A and B). This proves that salicylaldehyde can be used as a probe and can effectively compete with the various cellular targets for peroxynitrite, however, leaving the question whether this comes from peroxynitrite directly or peroxidase-catalyzed reaction. Previously, nitration of phenolic compounds such as tyrosine was also reported to proceed via (myelo)peroxidase-catalyzed reactions in the presence of hydrogen peroxide and inorganic nitrite [25,32,43,48–50]. Since peroxynitrite and peroxide/H_2O_2/nitrite reactivity leave the same footprint in biological systems, namely protein tyrosine nitration, there is ongoing discussion in the literature on the origin of nitrated proteins [25,26,29,32,48] with evidence of peroxynitrite being the essential/nitrating intermediate in the peroxide/H_2O_2/nitrite system [43] and molecular proof of differential contribution of these nitration pathways in response to different inflammatory stimuli [34]. Both nitration pathways are operative under inflammatory conditions due to the presence of immune cells with high peroxidase levels as well as NO/nitrite formation from inducible NO synthase as well as superoxide/hydrogen peroxide from the activated phagocytic NADPH oxidase [5,51,52]. Since previous (myelo)peroxidase inhibitors had to be used at rather high concentrations and had antioxidant properties (e.g. 4-aminobenzhydrazide) it was not easy to distinguish between peroxynitrite and peroxide/H_2O_2/nitrite reactivity, which will be improved with the recent development of highly specific and sensitive inhibitors of (myelo)peroxidase. With our last experiment, we also provide evidence for peroxide/H_2O_2/nitrite mediated nitration of salicylaldehyde, which however, only yields appreciable amount of products when supraphysiological concentrations of H_2O_2/nitrite were employed (Fig. 4C,D). An essential experiment in the future will be to quantify the nitration yield of salicylaldehyde in interferon-γ/lipopolysaccharide-stimulated J774A-1 macrophages, which are devoid of myeloperoxidase [53], in comparison with myeloperoxidase-containing immune cells. However, this study will probably require a more sensitive detection method for nitrated salicylaldehyde products such as electrochemical or mass spectrometric quantification.

Almost complete absence of nitrated salicylate products can be well explained by the low reactivity of salicylate for peroxynitrite-derived free radicals in comparison with other phenolic compounds such as tyrosine [54]. Despite the fact that salicylate is one of the most efficient scavengers of the hydroxyl radical [55], salicylate required 10-fold higher peroxynitrite concentrations to yield 50% nitration of the tyrosine reaction. Since oxidation of the aldehyde group in salicylaldehyde and simultaneous nitration in one combined reaction is highly unlikely, there will be either nitration in the first place, which will make the compound less reactive (due to steric and electron density reasons), or there will be conversion of the aldehyde to the carboxylic acid in the first place, which will result in a deactivated aromatic ring system, respectively. Likewise, another group reported that salicylate prevents NO/superoxide mediated lipid peroxidation under formation of dihydroxybenzoic acid but the employed Sin-1 concentration to generate peroxynitrite was very high (5 mM) [56], again highlighting the rather low reactivity of salicylate for peroxynitrite. To our best knowledge salicylaldehyde was never studied directly with respect to its reactivity for peroxynitrite, although metal complexes of salicylaldehyde efficiently prevented nitration of tyrosine and were proposed as SOD mimics [57]. In general, peroxynitrite was reported to react with aliphatic aldehydes to form various products via adduct formation in a first step, followed by free radical mechanism [58], supporting the here presented product yield (Fig. 5).

5. Conclusions

In order to adapt the here presented test for routine studies on peroxynitrite fluxes in biological systems the optical detection

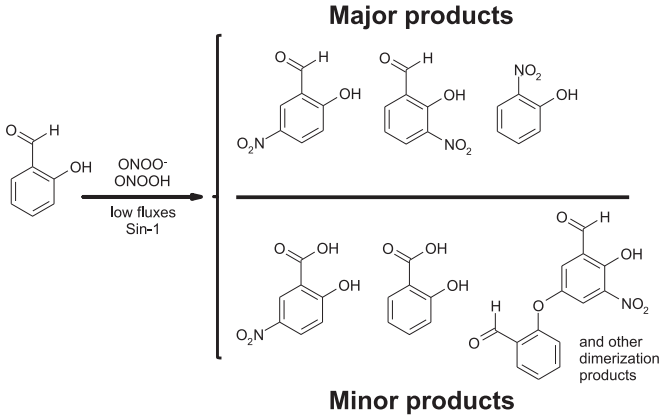


Fig. 5. Product yield of the reaction of salicylaldehyde and low fluxes of peroxynitrite by Sin-1. Of note, the major products are 2-nitrophenol and nitrated salicylaldehydes, whereas nitrated and non-nitrated salicylic acids as well as several dimerization products represent only minor reaction products.

used here can be replaced by electrochemical or mass spectrometric detection for an increase in sensitivity and specificity. By using 1 mM salicylaldeyde concentrations, the incubation of the samples can be performed over 60–120 min without losing essential amounts of the substrate by the ubiquitous presence of aldehyde oxidases and dehydrogenases under formation of salicylic acid. It is even likely that oxidases and dehydrogenases are physiologically inactivated under the formation of peroxynitrite since this seems to be one of the functions of peroxynitrite formation in the process of redox regulation. The reactive aldehyde group in salicylaldehyde probably serves as a major reaction center, similar to reaction described for carbon dioxide with peroxynitrite [59–62], leading to the formation of mono-nitrated, rather than dimerization products even at nanomolar peroxynitrite fluxes. This makes salicylaldehyde a unique phenolic reaction partner of peroxynitrite with special features preventing the previously reported highly abundant dimerization of other phenolic compounds at low fluxes of peroxynitrite.

Conflict of interest

none.

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