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Effect of ninhydrin on the biochemical and histopathological changes induced by ethanol in gastric mucosa of rats

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Abstract

Studies on the effect of ninhydrin in the normal gastric mucosa and against the ethanol induced gastric injury were undertaken in rats in view of the presence of a carbonyl function as well as hydroxyl groups in its chemical structure. In spite of its potentials to generate hydroxyl radicals, it is deemed to possess antioxidant property by virtue of its electrophillic nature. Recent studies have shown gastroprotection to mediate through a reaction between the electrophillic compounds and sulfhydryl groups of the mucosa. Hence it was found worthwhile to evaluate the interaction between the oxidant and antioxidant functions in the structure of the same compound. The effects of ninhydrin pretreatment on gastric mucosal injuries caused by 80% ethanol, 25% NaCl and 0.2M NaOH were investigated in rats. The gastric tissue in ethanol-treated rats was analyzed for different histopathological lesions. In addition, the effects on ethanol-induced changes in the gastric levels of proteins, nucleic acids, non-protein sulfhydryl (NP-SH) and malondialdehyde (MDA) were also evaluated. Ninhydrin, as such, failed to induce any significant changes in normal gastric mucosa, while its pretreatment at oral doses of 5, 10 and 20 mg/kg was found to provide a dose-dependent protection against the ulcers induced by ethanol, NaOH and NaCl. The results of histopathological evaluation revealed a protective effect of ninhydrin on congestion, hemorrhage, edema, erosions and necrosis caused by ethanol. Furthermore, the pretreatment afforded a dose-dependent inhibition of the ethanol-induced depletion of proteins, nucleic acids, NP-SH and increase of MDA in the gastric tissue. The results obtained clearly demonstrate the anti-ulcerogenic activity of ninhydrin. The exact mechanism of action is not known. However, the carbonyl function in ninhydrin appears to achieve antioxidant balance and protect the gastric mucosa from the ethanol-induced gastric injury. Further studies are warranted to investigate the toxicity and detailed mechanism of action of this potent compound before any clinical trials, especially at the effective lower doses.

Keywords: Ninhydrin; Gastroprotection; Histopathological and biochemical effects

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Introduction

Ninhydrin (2,2-dihydroxy-1,3-indanedione; CAS No. 485-47-2) is widely used as a reagent for the detection of free amino and carboxyl groups in proteins and peptides, in addition to the detection of some basic drugs and their metabolites by some specialized procedures. It represents a class of carbonyl compounds with a reactive keto group.

The chemical structure of ninhydrin (Fig. 1) shows it to be a carbonyl compound with reactive keto groups responsible for its hydrogen accepting property (1) and hydroxyl groups. Based on the chemical structure and its potential to generate hydroxyl radicals, ninhydrin has been described as an oxidant (2,3). Although, hydroxyl radicals are known to play a major role in the pathogenesis of gastric mucosal injury (4–6), the carbonyl compounds (as a whole) have been described to possess cytoprotective activity by virtue of their electrophillic nature. In a recent study (7), gastroprotection is described to be mediated through a reaction between the electrophillic compounds and sulfhydryl groups of the mucosa.

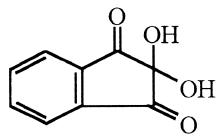
From a structure-activity point of view, ninhydrin appeared to possess both the ulcerogenic and anti-gastric ulcer properties. Hence it was found worthwhile to evaluate the effect of ninhydrin alone or as a pretreatment on the gastric mucosa and to study its effect on the biochemical and histopathological changes induced by ethanol in Wistar albino rats.

Materials and methods

Animal stocks: The animals' used and experimental design had the prior approval of the Animal Care and Use Committee, King Saud University, Saudi Arabia. Wistar albino male rats, bred at Experimental Animal Care Center, College of Pharmacy, Riyadh, Saudi Arabia, age 6–7 weeks and weighing 180–210g were used. The animals were maintained under standard conditions of humidity (40–45%), temperature (23–25°C) and light cycle (12h light, 12h dark) and were fed with Purina chow diet. Before the beginning of experiments, the animals were deprived of food for 36 h with free access to water.

Test compound, chemicals, reagents and treatment procedures: Ninhydrin (2,2-dihydroxy-1,3-indane dione; CAS No. 485-47-2) obtained from LKB Biochrom Ltd., Cambridge, England, was used as the test compound in the present study. All the other chemicals and reagents used in this study were of analytical reagent grade procured from commercial sources.

The doses were selected on the basis of 1/16th, 1/8th and 1/4th (5, 10 and 20 mg/kg), respectively of the LD_{50} (8) and a series of trial experiments, which showed cytoprotective activity of ninhydrin at a dose of 20 mg/kg. Aqueous solution of ninhydrin or tap water (vehicle) alone was administered intragastrically to the fasted rats.



Ninhydrin

Fig. 1.

Gastric lesion induction by different necrotizing agents: All the drugs and necrotizing agents were administered intragastrically at a constant volume of 1 ml per rat. The animals in the test groups were given different necrotizing agents (80% ethanol, 0.2M NaOH and 25% NaCl) which are known to produce gastric lesions (9,10). Hypertonic NaCl (25%) and NaOH (0.2M) were used only in cytoprotection studies. Ninhydrin was given 30 minutes before the necrotizing agents. The animals were killed under ether anesthesia, 1 h after treatment with the ulcerogenic agents. The stomach was excised and opened along the greater curvature. After washing with normal saline the gastric lesions were quantified using a binocular magnifier. The ulcers were scored according to the method of Valcavi et al. (11) and assessed on the basis of their dimensions. Deep circular ulcers more than 8 mm = 10; 7–8 mm = 8; 6–7 mm = 7; 5–6 mm = 6; 4–5 mm = 5; 3–4 mm = 4; 2–3 mm = 3; 1–2 mm = 2 and 0–1 mm = 1. The deep linear ulcer more than 10 mm in length = 6 and linear ulcer less than 10 mm in length = 3. The score for each single lesion were then summed up for the determination of ulcer index.

Histopathological procedures: The gastric tissue samples were preserved in 10% buffered formalin and processed for routine paraffin block preparation. Using an American Optical rotary microtome, sections of thickness about 5 μm were cut and stained with hematoxylin and eosin. These sections were examined under the microscope for histopathological changes such as congestion, hemorrhage, edema, erosions, and necrosis by an observer who was blind with respect to the treatment groups. The severity of these changes was expressed according to an arbitrary scale (12).

Determination of biochemical changes in ethanol-treated rats

Estimation of protein and nucleic acids: The levels of proteins and nucleic acids in the stomach tissue were determined according to the following procedures: The stomachs were rapidly dissected off the animals, frozen in liquid nitrogen and stored at −20°C until analyzed for total proteins and nucleic acids (RNA, DNA). Total proteins were determined by the method of Lowry et al. (13). The method described by Bregman (14) was used to determine the levels of nucleic acids. Tissues were homogenized and the homogenate was suspended in ice-cold trichloroacetic acid (TCA). After centrifugation, the pellet was leached with ethanol and then extracted with TCA by boiling in a water bath. DNA was determined by treating the nucleic acid extract with diphenylamine reagent and measuring the intensity of the blue color at 600nm. For quantification of RNA, the nucleic acid extract was treated with orcinol and the intensity of green color was measured at 660nm. Nucleic acids were quantified using the standard curves.

Estimation of non-protein sulfhydryls (NP-SH): Gastric mucosal NP-SH was measured according to the method of Sedlak and Lindsay (15). The glandular stomach was cut and homogenized in ice cold 0.02M ethylenediaminetetraaceticacid. The homogenate was mixed with 50% w/v aqueous TCA and centrifuged; the supernatants were mixed with Tris buffer, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) was added and the sample was shaken. The absorbance was read within 5 min of the addition of DTNB, at 412nm, against a reagent blank with no homogenate.

Malondialdehyde (MDA) estimation: The method described by Ohkawa et al. (16) was followed. The tissues were homogenized in TCA and homogenate was suspended in thiobar-

Table 1
Effect of ninhydrin pretreatment on the induction of gastric ulcers by various necrotizing agents in rats

Sl. No.	Treatment and dose (mg/kg)	Ulcer index (Mean ± S.E.)		
		80% Ethanol	0.2M NaOH	25% NaCl
1	Control (tap water)	28.8 ± 3.68	39.0 ± 1.89	20.6 ± 1.77
2	Ninhydrin (5)	14.8 ± 2.08*	23.0 ± 2.49**	09.8 ± 2.51*
3	Ninhydrin (10)	07.4 ± 1.17**	15.0 ± 2.34**	03.4 ± 1.29**
4	Ninhydrin (20)	03.2 ± 1.39**	09.2 ± 0.94**	02.2 ± 0.97**

Readings are Mean ± S.E. of five observations.
* $P<0.01$; ** $P<0.001$ (Student's t-test).

bituric acid. After centrifugation, the optical density of the clear pink supernatant was read at 532nm. Malondialdehyde bis-(dimethyl acetal) was used as a standard.

Results

The results of our present study are summarized in Tables 1–5 and Fig. 2 A-D.

Effect on normal gastric mucosa: Ninhydrin did not induce any significant changes in the normal gastric mucosa of rat at any of the doses tested.

Effect on the gastric lesions induced by different necrotizing agents: Pretreatment with ninhydrin was found to inhibit the gastric ulcers induced by different ulcerogenic agents (Table 1). The inhibition was statistically significant against ethanol at all the doses in a dose-dependent manner (5 mg/kg, $P<0.01$; 10 and 20 mg/kg, $P<0.001$). The pre-treatment with ninhydrin was also found to significantly inhibit the ulcers induced by sodium hydroxide ($P<0.001$) and hypertonic sodium chloride (5 mg/kg, $P<0.01$; 10 and 20 mg/kg, $P<0.001$).

Effect on histopathological gastric lesions: The treatment with ethanol caused considerable damage in the gastric mucosa. The histopathological lesions included congestion, edema, hemorrhage, erosions and necrosis. Pretreatment with ninhydrin provided significant and dose-dependent protection against the action of ethanol (Table 2; Fig. 2A–D).

Effect on protein and nucleic acid contents: The treatment with ethanol significantly reduced the protein ($P<0.01$) and nucleic acid ($P<0.001$) levels of gastric mucosa as compared to the control. Conversely, ninhydrin pretreatment significantly protected against the

Table 2
Effect of ninhydrin pretreatment on histopathological lesions induced in rat gastric mucosa after treatment with ethanol

Sl. No.	Treatment and dose (mg/kg)	Edema	Congestion	Hemorrhage	Erosions
1	Control (tap water)	—	—	—	—
2	Ethanol 80% (Et-OH)	+++	++++	+++	++++
3	Ninhydrin (5) + Et-OH	++	+++	++	++++
4	Ninhydrin (10) + Et-OH	+	+	—	+
5	Ninhydrin (20) + Et-OH	+	+	—	—

Five rats were used in each group.
—, Normal; +, Mild; ++, Moderate; +++, Severe; ++++, Intensively Severe.

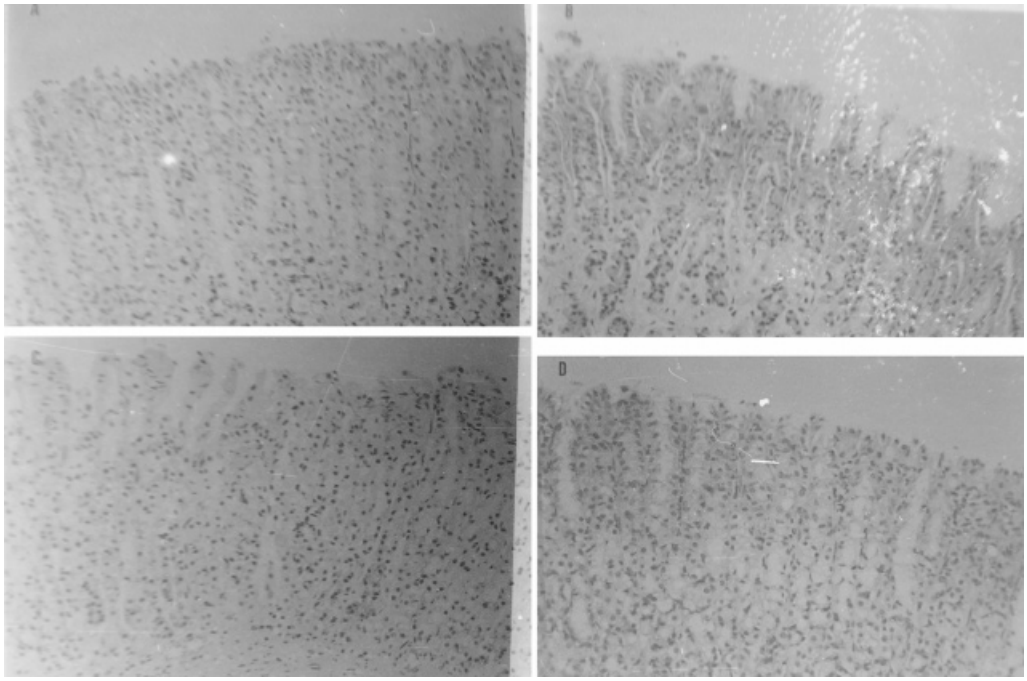


Fig. 2. Section through gastric mucosa of: A) a control rat showing its normal appearance (H & E ×125); B) a rat treated with 80% ethanol showing erosions, congestion, hemorrhage and edema (H & E ×125); C) a rat pretreated with ninhydrin (10 mg/kg) showing protection against ethanol-induced histopathological lesions except submucosal edema (H & E ×125); D) a rat pretreated with ninhydrin (10 mg/kg) showing protection against ethanol-induced histopathological lesions except superficial mild congestion of mucosal glands (H & E ×125).

inhibition of proteins (10 and 20 mg/kg, $P<0.05$), RNA (10 mg/kg, $P<0.01$; 20 mg/kg, $P<0.05$) and DNA (10 mg/kg, $P<0.01$; 20 mg/kg, $P<0.001$) seen in ethanol treated group (Table 3).

Effect on gastric mucosal levels of NP-SH: Ethanol treatment significantly (<0.001) reduced the NP-SH concentrations in the gastric mucosa as compared with the control. Ninhy-

Table 3
Effect of ninhydrin pretreatment on the protein and nucleic acid concentrations in gastric mucosa of rats treated with 80% ethanol (Et-OH)

Sl. No	Treatment and dose (mg/kg)	Proteins (mg/100 mg)	RNA (μg/100 mg)	DNA (μg/100 mg)
1	Control (tap water)	15.19 ± 0.32	605.44 ± 22.44	322.59 ± 14.04
2	Ethanol 80% (Et-OH)	13.41 ± 0.40**	394.20 ± 16.67***	218.82 ± 15.27***
3	Ninhydrin (5) + Et-OH	13.91 ± 0.21	454.32 ± 32.39	253.76 ± 6.21
4	Ninhydrin (10) + Et-OH	14.92 ± 0.34*	544.94 ± 39.15**	288.85 ± 11.78**
5	Ninhydrin (20) + Et-OH	14.51 ± 0.22*	533.32 ± 48.27*	315.32 ± 10.95***

Readings are determinations of mean ± S.E. of five samples in each group.
Group 2 was statistically compared with group 1 and groups 3, 4 and 5 were statistically compared with group 2.
* $P<0.05$; ** $P<0.01$; *** $P<0.001$ (Student's t-test).

Table 4
Effect of ninhydrin pretreatment on NP-SH contents in glandular stomach of rats treated by 80% ethanol

Sl. No	Treatment and dose (mg/kg)	No. of rats used	NP-SH contents (μmol/200 mg wet tissue)
1	Control (tap water)	5	2.60 ± 0.09
2	Ethanol 80% (Et-OH)	5	1.40 ± 0.19**
3	Ninhydrin (5) + Et-OH	5	1.45 ± 0.18
4	Ninhydrin (10) + Et-OH	5	1.75 ± 0.07
5	Ninhydrin (20) + Et-OH	5	2.05 ± 0.09*

Readings are determinations of mean ± S.E. of five determinations in a group.
Group 2 was statistically compared with group 1 and groups 3, 4 and 5 were statistically compared with group 2.
* $P<0.05$; ** $P<0.001$ (Student's t-test).

drin pretreatment significantly protected the NP-SH levels at the higher dose (20 mg/kg, $P<0.05$) as compared to the treatment with ethanol (Table 4).

Effect on MDA contents in gastric mucosa: The treatment with ethanol was found to significantly ($P<0.001$) increase the gastric mucosal levels of MDA. Pretreatment with ninhydrin significantly protected against the increase in the contents of MDA at all the doses (5 and 20 mg/kg, $P<0.05$; 10 mg/kg, $P<0.001$) as compared to the values obtained in the ethanol treated group (Table 5).

Discussion

The results of our present study confirmed the known ulcerogenic effects of ethanol, sodium hydroxide and hypertonic sodium chloride. The results on histopathology of gastric mucosa showed ethanol to induce congestion, hemorrhage, edema, erosions and necrosis. These data are supported by ethanol-induced depletion of the gastric levels of proteins, nucleic acids, NP-SH and an increase in contents of MDA. Our studies on the ulcerogenic potentials of ethanol confirm earlier observations that the depletion of NP-SH concentrations (17,18) and increase in the contents of free radicals (19–21) mediate tissue injury by stimulating lipid peroxidation and membrane damage by cross-linking proteins, lipids and nucleic acids (4–6).

Table 5
Effect of ninhydrin pretreatment on Malondialdehyde (MDA) contents in glandular stomach of rats treated by 80% ethanol

Sl. No	Treatment and dose (mg/kg)	No. of rats used	MDA Concentrations (nmol/g wet tissue)
1	Control (tap water)	5	199.96 ± 22.23
2	Ethanol 80% (Et-OH)	5	354.08 ± 16.99**
3	Ninhydrin (5) + Et-OH	5	273.42 ± 28.34*
4	Ninhydrin (10) + Et-OH	5	232.76 ± 11.61**
5	Ninhydrin (20) + Et-OH	5	279.10 ± 19.27*

Readings are mean ± S.E. of five determinations in a group.
Group 2 was statistically compared with group 1 and groups 3, 4 and 5 were statistically compared with group 2.
* $P<0.05$; ** $P<0.001$ (Student's t-test).

Pretreatment with ninhydrin confers a dose-dependent protection against gastric ulceration induced by ethanol, sodium hydroxide and hypertonic sodium chloride. The histopathological studies revealed it to inhibit congestion, hemorrhage, edema, erosions and necrosis induced by ethanol. These data clearly demonstrate the cytoprotective nature of ninhydrin (22) and are supported by its inhibitory effect on ethanol-induced depletion of protein, nucleic acids, NP-SH and increase in the levels of MDA in gastric mucosa. The ninhydrin-induced increase in the gastric tissue contents of NP-SH might have inhibited the impact of cytodestructive stimuli on the proteins, nucleic acids and MDA, possibly by an elevation of gamma-glutamyl transpeptidase (GGT) (23). Previous reports have also shown etiology of gastric ulcer to be closely related to the gastric mucosal GSH and GSH-dependent enzymes (24).

The chemical structure (Fig. 1) shows ninhydrin to be a carbonyl compound with reactive keto groups responsible for its hydrogen accepting property (1) and hydroxyl groups. Based on its structure and potentials to generate hydroxyl radicals ninhydrin has been described to be an oxidant (2,3) and appears to increase the load of free radicals. Nevertheless, it is interesting to note that the carbonyl compounds (as a whole) have been described to possess cytoprotective activity by virtue of their electrophillic acceptor nature. In presence of a non-hindered electrophillic acceptor in the structure, the mechanism of cytoprotective activity has been suggested to be (at least, in part) mediated through a reaction between the electrophillic acceptor and the sulfhydryl-containing groups of the mucosa. Thus the carbonyl group in ninhydrin appears to achieve the antioxidant balance and protect the gastric mucosa from the injurious agents (4). The gastroprotection observed in the present study may be due to the electrophillic acceptor nature of ninhydrin. The mode of inhibition of gastric ulcers by ninhydrin appears to be an oxidation of functionally essential SH groups of the mucosa, where the reactive keto group of the inhibitor acts as the hydrogen acceptor (1,25,26). Rodriguez et al. (7), in a recent study, also found gastroprotection to be mediated through a reaction between the electrophillic acceptor and sulfhydryl groups of the mucosa. The exact mechanism of action is not known, however it appears to be the antioxidant activity of the carbonyl function. In a previous report on the structure-scavenging activity relationship of rebamipide (a novel anti-peptic ulcer agent) and seven related compounds, the carbonyl portion of the amido group has been focussed as a substantial determinant for the scavenging of hydroxyl radicals (27). Further studies are warranted to investigate the toxicity and detailed mechanism of action of this potent compound before any clinical trials, especially at the effective lower doses.

References

1. S. LENZEN, F.H. BRAND, U. PANTEN, Br. J. Pharmacol. **95** 851–859 (1988).
2. G. KOKKINI, V.J. STEVENS, C.M. PETERSON, A. CERAMI, Blood **56** 701–705 (1980).
3. K. GRANKVIST, J. SEHLIN, I.B. TALJEDAL, Acta-Pharmacol-Toxicol-Copenh. **58** 175–181 (1986).
4. T. OTAMIRI, R. SJODAHL, Dig. Dis. **9** 133–141 (1991).
5. Y. NAITO, T. YOSHIKAWA, K. MATSUYAMA, N. YAGI, M. ARAI, Y. NAKAMURA, S. NISHIMURA, N., N. YOSHIDA, M. KONDO, J. Clin. Gastroenterol. **21** Suppl 1 S82–86 (1995).
6. K. WADA, Y. KAMISAKI, M. KITANO, Y. KISAHIMOTO, K. NAKAMOTO, T. ITOH, Pharmacology, **54** 57–63 (1997).
7. A.M. RODRIGUEZ, R.D. ENRIZ, L.N. SANTAGATA, E.A. JAUREGUI, M.J. PESTCHANKER, O.S. GIORDANA, J. Med Chem. **40** 1827–34 (1997).

8. M. WINDHOLZ, S. BUDAVARI, R.F. BLUMETTI, E.S. OTTERBEIN, *The Merck Index*, Merck and Co. Inc. New Jersey, USA, pp 6395 (1983).
9. A. ROBERT, J.E. NEZAMIS, C. LANCASTER, J.P. DAVIS, S.O. FIELD, A.J. HANCHAR, Am. J. Physiol. **245** G113–G121 (1983).
10. A.M. AL-BEKAIRI, S. QURESHI, M.M. AHMED, M. AFZAL, A.H. SHAH, Food Chem. Toxicol. **30** 525–531 (1992).
11. V. VALCAVI, R. CAPONI, A. BRAMBILLA, F. PALMIRA, F. MONOJA, F. BERNINI, R. MUSTANI, R. FUMAGALL, Arzneim. Forsch. **32** 657–663 (1982).
12. M.M. AL-HARBI, S. QURESHI, M. RAZA, M.M. AHMED, A.B. GIANGRECO, A.H. SHAH, Eur. J. Cancer Prevent. **4** 307–318 (1995).
13. O.H. LOWRY, N.S. ROSENBROUGH, A.L. FARR, R.J. RANDALL, J. Biol. Chem. **193** 265–275 (1951).
14. A.A. BREGMAN, In *Laboratory Investigations and Cell Biology*, pp. 51–60, Wiley, New York (1983).
15. J. SEDLAK, AND R.H. LINDSAY, Anal. Biochem. **25** 192–195 (1968).
16. H. OHKAWA, N. OHISHI, K. YAGI, Anal. Biochem. **95** 351–358 (1979).
17. N.S. KOSOWER, AND E.M. KOSOWER, Internal Rev. Cytol. **54** 109–160 (1978).
18. S. SZABO, J.S. TRIER, A. BROWN, J. SCHNOOR, H.D. HOMAN, J.C. BRADFORD, J. Pharmacol. Methods, **13** 59–66 (1985).
19. J.D. BUTTERFIELD, C.P. MCGRAW, Stroke, **9** 443–445 (1978).
20. R.F. DEL MAESTRO, H.H. THAW, J. BJORK, M. PLANKER, K.E. ARFORS, Acta Physiol. Scand. (Suppl.) **492** 43–57 (1980).
21. A.S. SALIM, The Am. J. Med. Sci. **300** 1–6 (1990).
22. A. ROBERT, Gastroenterology, **77** 761–767 (1979).
23. Y. SHUKLA, M. ANTONY, K.P. GUPTA, N.K. MEHROTRA, Food Chem Toxicol. **322** 651–654 (1994).
24. K. HIROKAWSA, H. KAWASAKI, Res. Commun. Mol. Pathol. Pharmacol. **88** 163–176 (1995).
25. S. LENZEN, S. FREYTAG, U. PANTEN, Mol. Pharmacol. 0**34** 395–400 (1988).
26. M. TIEDGE, U. KRUG, S. LENZEN, Biochim Biophys Acta **1337** 175–90 (1997).
27. Y. NAITO, T. YOSHIKAWA, T. TANIGAWA, K. SAKURAI, K. YAMASAKI, M. UCHIDA, M. KONDO, Free Radic. Biol. Med. **18** 117–123 (1995).