ANTIOXIDANTS & REDOX SIGNALING
Volume 4, Number 1, 2002

Rapid Letter

N-Acetylcysteine Negatively Modulates Nitric Oxide Production in Endotoxin-Treated Rats Through Inhibition of NF-κB Activation

CRISTINA ROTA,[1] STEFANIA BERGAMINI,[1] FRANCESCA DANERI,[1] ALDO TOMASI,[1] FABIO VIRGILI,[2] and ANNA IANNONE[1]

ABSTRACT

***N*-Acetylcysteine (NAC) has a wide spectrum of biological activities, either related to the ability to increase intracellular thiols or directly acting as an antioxidant. We used an *in vivo* animal model to study NAC modulation of nitric oxide (NO) production in response to lipopolysaccharide treatment. A comparison was made between NAC and the *N*-[3-(aminomethyl)benzyl] acetamidine (1400W), an inhibitor of the inducible NO synthase (iNOS). Both inhibit NO production, although NAC lacks any effect if given when iNOS is already induced; this indicates that the decrease of NO generation is not due to an effect on iNOS activity. We found that the DNA binding activity of nuclear transcription factor-κB in peripheral blood cells was inhibited by NAC given before lipopolysaccharide, whereas tumor necrosis factor-α secretion was not affected.** Antioxid. Redox Signal. 4, 221–226.

INTRODUCTION

N-Acetylcysteine (NAC) is a sulfhydryl group donor that crosses the cell membrane and restores glutathione (GSH) levels by providing cysteine for its synthesis. Accordingly, NAC has been reported to block nuclear factor-kB (NF-kB) activation induced in cells by the addition of hydrogen peroxide (10, 11), corroborating the relationship between cellular redox status, thiol levels, and intracellular signaling.

The lipopolysaccharide (LPS) is known to induce an increase of prooxidant species. In the macrophages, it induces the secretion of several cytokines, with subsequent generation of prooxidant molecules, activation of the NF-κB pathway, finally leading to the expression of the inducible form of nitric oxide synthase (iNOS). Following the reaction of the produced nitrogen monoxide (NO) with simultaneously generated superoxide, highly reactive nitrogen-containing species are formed. This situation leads to an imbalance of the redox cellular homeostasis. We have already reported that LPS administration to rats is a valid experimental model to study acute iNOS induction *in vivo* (1). We also reported that pretreatment with NAC of LPS-administered rats was associated with a decrease in plasma nitrosyl-hemoglobin (NO-Hb).

[1]Department of Biomedical Sciences, University of Modena and Reggio Emilia, 41100 Modena, and [2]National Institute for Food and Nutrition Research, 00178 Rome, Italy.

Here we report the comparison between NAC and the iNOS inhibitor *N*-[3-(aminomethyl)benzyl]acetamidine (1400W), to demonstrate that NAC does not interact directly with iNOS activity in LPS-treated rats.

MATERIALS AND METHODS

Animals and treatments

Male Wistar rats (150–200 g) were kept at 21 ± 1°C and 60% humidity, on a light-dark cycle, with food and water *ad libitum.* Housing conditions and experiments were in accordance with the European Community regulations (EC Council 86/609, and D.L. 27/01/92, no. 116). Rats were acclimatized for at least 1 week before being used and fasted 24 h at the time of the experimental procedure.

LPS from *Escherichia coli* (serotype 055:B5; Sigma–Aldrich, Milan, Italy) was prepared in sterile saline and given intraperitoneally (20 mg/kg). Some rats were also given 150 mg/kg i.p. NAC (Zambon Italia) in sterile saline, 30 min before or 3 h after the LPS injection. Another group was treated with a subcutaneous injection of 1400W (5 mg/kg) 30 min before or 3 h after the LPS injection.

Control groups received (a) saline (two injections), (b) saline and NAC (at −30 min and 3 h, respectively), (c) NAC and saline (at −30 min and 3 h), (d) saline and 1400W (at −30 min and 3 h), or (e) 1400W and saline (at −30 min and 3 h).

Six hour after the LPS injection, rats were anesthetized by diethyl ether inhalation. Blood was collected in heparinized syringes; 1 ml was collected in an EDTA-containing syringe. The heparinized blood was centrifuged to separate plasma for citrulline and nitrite assays. The red blood cells were used for NO-Hb measurement; they were placed in a quartz tube, frozen in liquid nitrogen, and stored at −80°C, until the electron spin resonance (ESR) measurement. The EDTA-treated blood was also centrifuged to separate plasma, added with aprotinin, and stored at −80°C until enzyme-linked immunosorbent assay (ELISA) for tumor necrosis factor-α (TNF-α) was performed.

A separate set of experiments was performed to measure NF-κB binding activity in isolated peripheral blood mononuclear cells (PBMCs); animals were treated with LPS and/or NAC and killed 90 min after the endotoxin administration. PBMCs were isolated as previously described (1).

Measurements of NO-Hb, citrulline, nitrite/nitrate, and TNF-α

NO-Hb, citrulline, and nitrite were measured as previously described (1). For TNF-α detection, EDTA-treated plasma was added with aprotinin to inhibit protease inactivation of the cytokine; 0.02 trypsin inhibitory unit of aprotinin/ml of plasma was necessary. An ELISA kit (Endogen, Inc., Woburn, MA, U.S.A.) was used. A standard curve was performed by using rat TNF-α.

Electromobility shift assay (EMSA) for NF-κB binding activity

Nuclear extracts from PBMCs were prepared according to Suzuki and Packer (12). Two double-stranded deoxyoligonucleotides were end-labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; NEN Life Science Products, Boston, MA, U.S.A.) using a T4 kinase (Amersham Pharmacia Biotech, Uppsala, Sweden). Nuclear extracts (10 μg) were preincubated for 15 min at 2°C with 2 μg of poly(dI-dC) (Pharmacia) and incubated with ^{32}P-labeled DNA NF-κB probe in binding buffer (25 m*M* HEPES, 5 m*M* $MgCl_2$, 50 m*M* NaCl, 1 m*M* dithiothreitol, 10% glycerol, pH 7.8) for 20 min at room temperature. DNA binding activity was separated from the free probe using 5% polyacrylamide gel in 0.5 × Tris–borate–EDTA buffer, run at 200 V. Following electrophoresis, the gel was dried and autoradiographed. The specificity of the NF-κB band was checked by incubating the positive control with 20-fold excess of nonlabeled probe.

RESULTS

LPS treatment leads to the expression of iNOS, which is downstream from the activa-

tion of response factors such as NF-κB. iNOS generates sustained levels of NO rapidly diffusing in the blood. The measurement of nitrite and nitrate in plasma is a reliable method to confirm NO overproduction *in vivo*, although confounding factors, such as intestinal sources of nitrogen species entering the circulation stream as a consequence of LPS-induced intestinal injury, may affect the interpretation of the data. Hence we used three different ways to measure NO, including ESR analysis of the NO-Hb adduct.

Six hours after LPS treatment (20 mg/kg i.p.), the ESR signal related to NO-Hb was well evident, indicating the generation of NO and the subsequent reaction with the heme group of deoxyhemoglobin (Fig. 1A). Concurrently, nitrites in plasma of LPS-treated animals significantly increased (Fig. 1B). The concentration of citrulline, the by-product of NO synthesis from L-arginine breakdown, followed the same pattern and increased within the same time window, indicating an induction of *de novo* synthesis (Fig. 1C).

When animals were treated with NAC (150 mg/kg i.p.) 30 min before LPS, these parameters were significantly and negatively affected (Fig. 1), indicating that NO synthesis is inhibited. On the other hand, when NAC was administered 3 h following LPS injection, NO generation was not affected. Surprisingly, though, the citrulline levels were significantly higher with respect to the group of rats injected with LPS alone (Fig. 1C). Overall, these data indicate that NO inhibition is obtained by NAC acting on the transactivation of the iNOS gene, and not as an inhibitor of the enzyme itself.

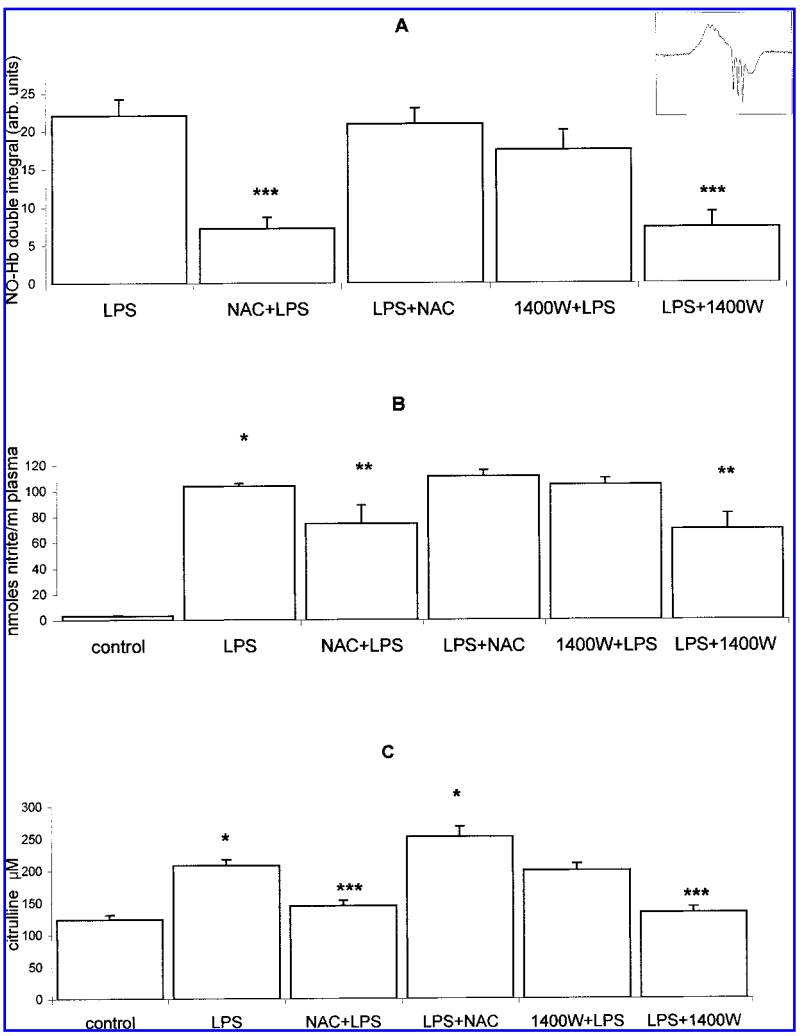

FIG. 1. Effect of NAC and 1400W on NO-Hb, nitrite/nitrate, and citrulline in rats injected with LPS. All the animals were killed 6 h after the LPS treatment; NAC or 1400W was administered either 30 min before or 3 h after the endotoxin (as indicated). The control group was injected with saline. Results (means ± SE) represent the average of seven to 10 rats (*$p < 0.01$, vs. control; **$p < 0.05$ and ***$p < 0.01$, vs. LPS). (**A**) NO-Hb levels (the ESR signal in the **inset**). Red blood cell samples from control rats in each group were negative for NO-Hb detection. (**B**) Nitrite levels in plasma. (**C**) Plasma citrulline levels.

To clarify this point further, we administered subcutaneously the specific iNOS inhibitor 1400W (5 mg/kg), 30 min before or following the LPS challenge. 1400W is a molecule that tightly binds and selectively inhibits iNOS both *in vivo* and *in vitro* (6, 7, 13). A significant inhibition of NO generation, as assessed by determination of NO-Hb, nitrite, and citrulline formation, was observed only when 1400W was given 3 h after the endotoxic stimulus (Fig. 1). No effect on NO production was detected when 1400W was administered before LPS. This differential activity on the basis of the treatment timing suggests that the half-life of this molecule is probably <3 h, which corresponds to the peak time of iNOS expression and NO generation due to the endotoxic stimulus. This pattern of activity, clearly different from that observed for the NAC treatment, indicates that the thiol compound does not directly influence iNOS activity.

The neosynthesis of iNOS induced by LPS depends on the activation of tyrosine kinase and on the nuclear DNA binding by the transcription factor NF-κB heterodimers p50/c-Rel and p65/RelA (15). In turn, the production of NO inhibits NF-κB binding to DNA (8, 9), thus acting as a feedback regulator of the overall NO flow. We have already reported that iNOS-mRNA expression in PBMCs from LPS-treated rats is inhibited by NAC treatment (1).

Therefore, we sought to investigate whether the observed decrease of NO flow, associated with NAC administration in LPS-treated animals, is due to an effect at the transcriptional level. As it is known that iNOS inhibitors augment the NF-κB binding activity to DNA through the lack of negative feedback produced by NO (2, 4, 14), we performed the NF-κB assay 90 min after LPS intoxication, when the NO levels are still undetectable in blood (1). Figure 2 shows the EMSA for NF-κB transcription factor performed on the nuclear protein extracts of PBMCs collected from LPS-injected rats. In basal conditions, NF-κB binding activity was almost undetectable in PBMCs (Fig. 2, lane 1). After endotoxin stimulus, the binding of RelA-proteins to the DNA consensus sequence significantly increased (Fig. 2, lane 2), eventually leading to the transactivation of iNOS gene. When rats were treated with NAC, the NF-κB binding activity was significantly inhibited (Fig. 2, lanes 4 and 5) with respect to the levels observed after LPS injection alone. NAC treatment alone had no effect on NF-κB binding activity (Fig. 2, lane 3). This observation indicates that NAC acts at the transcriptional level, possibly inhibiting some of the steps upstream from NF-κB activation and transfer to the cell nucleus.

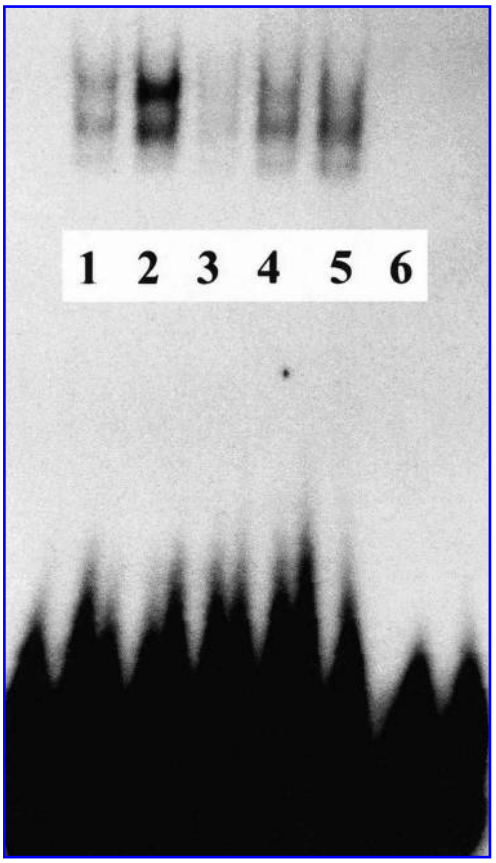

FIG. 2. NAC inhibits NF-κB activation in PBMCs from LPS-treated rats. NF-κB DNA binding activity of the nuclear extract of PBMCs obtained from LPS-treated rats is shown. Lane 1, control animal; lane 2, rat treated with LPS; lane 3, rat treated with NAC; lanes 4 and 5, rats treated with both NAC and LPS; and lane 6, as in lane 2 plus cold competitor. One representative experiment of three is presented.

The signaling and response cascade following the LPS stimulus on PBMCs acts through the intervention of TNF-α. To evaluate if the observed inhibition of NF-κB binding activity is due, at least in part, to a decrease of TNF-α-mediated response, we measured the levels of

TABLE 1. EFFECT OF NAC ON PLASMA LEVELS OF TNF-α IN LPS-TREATED RATS

Groups of treatment	*pg/ml TNF-α*
Control	18.5 ± 15.2 (4)
NAC	0 ± 0 (3)
LPS	93.7 ± 17.7 (5)*
NAC + LPS	72.5 ± 2.1 (9)
LPS + NAC	135.6 ± 63 (4)

LPS was alwyas administered 6 h before animals were killed and NAC 30 min before LPS (NAC + LPS group) or 3 h after LPS treatment (LPS + NAC group). The values are means ± SE (number of experiments). *$p < 0.05$, vs. control.

this cytokine in plasma collected from animals 6 h after treatment with either LPS alone or LPS plus NAC. It is known that the plasma levels of this cytokine increase in the rat within 1 h following the administration of 1 μg/kg i.v. of LPS (3). In our model system, we found that TNF-α levels persist at a relatively high level up to 6 h after the administration of a larger intraperitoneal dose of LPS (Table 1). At this time, we evaluated the effect of NAC, administered 30 min before or 3 h after the endotoxin; this thiol did not significantly affect TNF-α levels, indicating that the inhibition of NF-κB binding activity by NAC is independent of TNF-α secretion and is possibly due to its intervention in the redox-sensitive pathway of NF-κB activation.

DISCUSSION

We have recently reported on LPS administration in the rat as an experimental model to study acute overproduction of NO by iNOS and to evaluate the effect of NAC on this response (1).

To understand better the molecular basis of the inhibitory effect of NAC on NO generation in septic shock, in this study we investigated both the possibility of a direct inhibition of iNOS enzyme activity and its effect on the signaling cascade leading to iNOS expression. For this purpose, the effects of NAC and 1400W on iNOS were compared.

We administered NAC either before or after LPS treatment. The different effect observed after the administration of the iNOS inhibitor 1400W points out that NAC does not directly inhibit iNOS enzyme activity.

We sought to understand further at what level NAC is affecting the signaling cascade between endotoxic stimulus and iNOS-mRNA expression. It is known that LPS-induced expression of iNOS is dependent on the activation of tyrosine kinase and on the binding of NF-κB to DNA (5). We have demonstrated that activation of this transcriptional factor occurs also *in vivo* following endotoxin treatment, as indicated by the significant increase of NF-κB binding activity to the consensus sequence, tested by EMSA in PBMCs. The administration of NAC significantly decreases the response induced by LPS.

On the other hand, TNF-α secretion is not affected by NAC. This indicates that the primary response to LPS is not thiol-regulated and suggests that the block by NAC of the signaling cascade activated by LPS could be due rather to a combination of the following activities: (a) The thiol donor activity of NAC improves the cellular antioxidant network and down-regulates the redox-sensitive mechanisms of NF-κB activation. This effect can be achieved either by providing the fuel for the *ex novo* GSH synthesis or by increasing the thiol intracellular levels. (b) There may exist a specific activity on a step upstream from the release of the inhibitory subunit I-κB, such as the maintenance at reduced status of critical cysteine residual on the protein skeleton.

ACKNOWLEDGMENTS

This work was supported by MURST (Ministero dell'Università e della Ricerca Scientifica e Tecnologica) and Zambon Group, Bresso (Milan), Italy. The authors thank Dr. Luciano Licciardello of the Zambon Group for helpful discussions.

ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; EMSA, electromobility shift assay; ESR, electron spin resonance; GSH, glutathione; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NAC, *N*-acetylcysteine;

NF-κB, nuclear factor κB; NO, nitric oxide; NO-Hb, nitrosyl hemoglobin; PBMCs, peripheral blood mononuclear cells; TNF-α, tumor necrosis factor-α; 1400W, *N*-[3-(aminomethyl)-benzyl]acetamidine.

REFERENCES

1. Bergamini S, Rota C, Canali R, Staffieri M, Daneri F, Bini A, Giovannini F, Tomasi A, and Iannone A. *N*-Acetylcysteine inhibits in vivo nitric oxide production by inducible nitric oxide synthase. *Nitric Oxide* 5: 349–360, 2001.
2. Chen F, Lu Y, Castranova V, Rojanasakul Y, Miyahara K, Shizuta Y, Vallyathan V, Shi X, and Demers LM. Nitric oxide inhibits HIV tat-induced NF-kappaB activation. *Am J Pathol* 155: 275–284, 1999.
3. Choi SH, Cho JY, Chung YS, Hong E, Han Y, and Kim SG. Inhibition of lipopolysaccharide-induced I-kappaB degradation and tumor necrosis factor-alpha expression by acriflavine, an antimicrobial agent. *Int J Immunopharmacol* 22: 775–787, 2000.
4. DelaTorre A, Schroeder RA, Punzalan C, and Kuo PC. Endotoxin-mediated *S*-nitrosylation of p50 alters NF-kappa B-dependent gene transcription in ANA-1 murine macrophages. *J Immunol* 162: 4101–4108, 1999.
5. Gabbita SP, Robinson KA, Stewart CA, Floyd RA, and Hensley K. Redox regulatory mechanisms of cellular signal transduction. *Arch Biochem Biophys* 376: 1–13, 2000.
6. Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJ, and Knowles RG. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. *J Biol Chem* 272: 4959–4963, 1997.
7. Hamilton LC and Warner TD, Interactions between inducible isoforms of nitric oxide synthase and cyclooxygenase in vivo: investigations using the selective inhibitors, 1400W and celecoxib. *Br J Pharmacol* 125: 335–340, 1998.
8. Matthews JR, Botting CH, Panico M, Morris HR, and Hay RT. Inhibition of NF-kappaB DNA binding by nitric oxide. *Nucleic Acids Res* 24: 2236–2242, 1996.
9. Peng HB, Libby P, and Liao JK. Induction and stabilization of I kappa B alpha by nitric oxide mediates inhibition of NF-kappa B. *J Biol Chem* 270: 14214–14219, 1995.
10. Schreck R, Rieber P, and Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J* 10: 2247–2258, 1991.
11. Schreck R, Albermann K, and Baeuerle PA. Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic Res Commun* 17(4): 221–237, 1992.
12. Suzuki YJ and Packer L. Inhibition of NF-kappa B activation by vitamin E derivatives. *Biochem Biophys Res Commun* 193: 277–283, 1993.
13. Thomsen LL, Scott JM, Topley P, Knowles RG, Keerie AJ, and Frend AJ. Selective inhibition of inducible nitric oxide synthase inhibits tumor growth in vivo: studies with 1400W, a novel inhibitor. *Cancer Res* 57: 3300–3304, 1997.
14. Walley KR, McDonald TE, Higashimoto Y, and Hayashi S. Modulation of proinflammatory cytokines by nitric oxide in murine acute lung injury. *Am J Respir Crit Care Med* 160: 698–704, 1999.
15. Xie QW, Kashiwabara Y, and Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem* 269: 4705–4708, 1994.

Address reprint requests to:
Anna Iannone
Department of Biomedical Sciences
University of Modena and Reggio Emilia
Via Campi 287
41100 Modena, Italy

e-mail: iannone@unimo.it

Received for publication November 7, 2001; accepted November 12, 2001.

This article has been cited by:

1. André Luis Casarin, Maria Elisa Lopes-Pires, Rafael Prada Morganti, Edson Antunes, Sisi Marcondes. 2011. Reactive oxygen and nitrogen species modulate the ex-vivo effects of LPS on platelet adhesion to fibrinogen. *Life Sciences* . [CrossRef]
2. Anna Iannone, Anna Petroni, Elisabetta Murru, Lina Cordeddu, Gianfranca Carta, Maria Paola Melis, Stefania Bergamini, Lara Della Casa, Laura Cappiello, Romina Carissimi. 2009. Impairment of 8-iso-PGF2ALPHA isoprostane metabolism by dietary conjugated linoleic acid (CLA). *Prostaglandins, Leukotrienes and Essential Fatty Acids* **80**:5-6, 279-287. [CrossRef]
3. Gustavo Cumbo-Nacheli, Jarrett Weinberger, Michel Alkhalil, Neelima Thati, Alan P. Baptist. 2008. Anticonvulsant hypersensitivity syndrome: Is there a role for immunomodulation?. *Epilepsia* **49**:12, 2108-2112. [CrossRef]
4. Demeral David Liu, Shang-Jhy Kao, Hsing I. Chen. 2008. N-Acetylcysteine attenuates acute lung injury induced by fat embolism*. *Critical Care Medicine* **36**:2, 565-571. [CrossRef]
5. Yasutaka Ikeda, Akira Murakami, Hajime Ohigashi. 2008. Ursolic acid: An anti- and pro-inflammatory triterpenoid. *Molecular Nutrition & Food Research* **52**:1, 26-42. [CrossRef]
6. Claudia Pinto Marques Souza de Oliveira, José Tadeu Stefano, Erika Rabelo Forte de Siqueira, Leonardo Soares Silva, Daniel Ferraz de Campos Mazo, Vicência Mara Rodrigues Lima, Carlos Kioshi Furuya, Evandro Sobroza Mello, Fabrício G. Souza, Fabíola Rabello, Telma Eugênio Santos, Monize Aydar Nogueira, Stephen H. Caldwell, Venâncio Avancini Ferreira Alves, Flair José Carrilho. 2007. Combination of N-acetylcysteine and metformin improves histological steatosis and fibrosis in patients with non-alcoholic steatohepatitis. *Hepatology Research*, ahead of print070809084409001-???. [CrossRef]
7. Xue-Yun Zhang, Seiji Hayasaka, Yoriko Hayasaka, Hu-Shan Cui, Zai-Long Chi. 2007. Effect of N-Acetylcysteine on Lipopolysaccharide-Induced Uveitis in Rats. *Japanese Journal of Ophthalmology* **51**:1, 14-20. [CrossRef]
8. Dur-Zong Hsu, Ya-Hui Li, Pei-Yi Chu, Se-Ping Chien, Yin-Ching Chuang, and Ming-Yie Liu. 2006. ATTENUATION OF ENDOTOXIN-INDUCED OXIDATIVE STRESS AND MULTIPLE ORGAN INJURY BY 3,4-METHYLENEDIOXYPHENOL IN RATS. *Shock* **25**:3, 300-305. [CrossRef]
9. Shang Jyh Kao, David Wang, Hen I Lin, Hsing I Chen. 2006. N-ACETYLCYSTEINE ABROGATES ACUTE LUNG INJURY INDUCED BY ENDOTOXIN. *Clinical and Experimental Pharmacology and Physiology* **33**:1-2, 33-40. [CrossRef]
10. Zhengyuan Xia, Prabhakara R. Nagareddy, Zhixin Guo, Wei Zhang, John H. Mcneill. 2006. Antioxidant N -acetylcysteine restores systemic nitric oxide availability and corrects depressions in arterial blood pressure and heart rate in diabetic rats. *Free Radical Research* **40**:2, 175. [CrossRef]
11. X CHENG, Z XIA, J LEO, C PANG. 2005. The effect of -acetylcysteine on cardiac contractility to dobutamine in rats with streptozotocin-induced diabetes. *European Journal of Pharmacology* **519**:1-2, 118-126. [CrossRef]
12. Damian Vassilev, Balázs Hauser, Hendrik Bracht, Zsolt Iványi, Michael Schoaff, Pierre Asfar, Josef Vogt, Ulrich Wachter, Hubert Schelzig, Michael Georgieff, Uwe B. Brückner, Peter Radermacher, Gebhard Fröba. 2004. Systemic, pulmonary, and hepatosplanchnic effects of N-acetylcysteine during long-term porcine endotoxemia*. *Critical Care Medicine* **32**:2, 525-532. [CrossRef]
13. Han#Jung Chae, Soo#Wan Chae, Hyung#Ryong Kim. 2004. N#Acetyl Cysteine Regulates TNF### Inhibited Differentiation in ROS 17/2.8 Osteoblasts. *Immunopharmacology and Immunotoxicology* **26**:2, 203-213. [CrossRef]
14. Elisa Bellei, Cristina Rota, Stefania Bergamini, Paolo Manfredini, Alberto Albertazzi, Aldo Tomasi, Anna Iannone. 2004. Effect of #-tocopherol andN-acetylcysteine on benzoyl peroxide toxicity in human keratinocytes. *Journal of Biochemical and Molecular Toxicology* **18**:2, 107. [CrossRef]

15. SEVAL AYDIN, RESAT OZARAS, HAFIZE UZUN, AHMET BELCE, EZEL USLU, VEYSEL TAHAN, TUNCAY ALTUG, EMEK DUMEN, HAKAN SENTURK. 2002. N-Acetylcysteine Reduced the Effect of Ethanol on Antioxidant System in Rat Plasma and Brain Tissue. *The Tohoku Journal of Experimental Medicine* **198**:2, 71-77. [CrossRef]