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Antioxidant activity of endogenously produced nitric oxide against the zinc oxide nanoparticle-induced oxidative stress in primary hepatocytes of air-breathing catfish, *Clarias magur*

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ABSTRACT

The facultative air-breathing magur catfish (*Clarias magur*) regularly encounter various environmental challenges including the exposure to nanomaterials discarded as industrial wastes in water bodies. The present investigation aimed at determining the possible ZnO NP-induced oxidative stress and also the antioxidant strategy of nitric oxide (NO), generated endogenously, in primary hepatocytes of magur catfish. Exposure of primary hepatocytes to different concentrations of ZnO NPs (5 and 10 μg/mL) led to a sharp rise of intracellular concentrations of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) within 6 h, which decreased gradually at later stages. This phenomenon was accompanied by an initial decrease of superoxide dismutase (SOD) and catalase (CAT) activities, the expression of their corresponding genes and the enzyme protein levels, with a subsequent significant increase of all these parameters at later stages. Most interestingly, exposure to ZnO NPs also stimulated the NO production by the primary hepatocytes as a consequence of induction of inducible nitric oxide synthase (iNOS) activity, higher expression of *nos2* gene and iNOS protein. Furthermore, when the NO production by the hepatocytes was inhibited by either aminoguanidine (inhibitor for iNOS) or BAY (inhibitor for NFκB) in the presence of ZnO NPs, the intracellular concentrations of H_2O_2 and MDA was significantly elevated. This elevation was accompanied by a subsequent decrease of *sod* and *cat* genes expression, thereby suggesting that the inhibition of NO production leads to oxidative stress. Thus, it is believed that the magur catfish uses the strategy of stimulation of endogenous NO production by inducing the *nos2* gene and simultaneous NO-mediated induction of *sod* and *cat* genes to defend against the NP-induced oxidative stress. It is the first report of such NO-mediated antioxidant strategy in any teleost fish to defend against the NP-induced oxidative stress and corresponding cellular damages.

1. Introduction

In recent years, studies on the potential roles of nanoparticles (NPs) in causing cellular damages in the biological system have gained much interest. Zinc oxide (ZnO) NPs are the most extensively used NPs in the industry as well as personal care products because of their high stability, anticorrosion and photocatalytic properties [1]. At the same time, ZnO NPs are known to exhibit the highest cytotoxicity about cell proliferation, cell viability and membrane integrity in cell lines [2]. There are also reports on ZnO NP-induced reactive oxygen species (ROS) generation in different cell lines [3–6]. Animals are known to possess various enzymatic and non-enzymatic antioxidant defense mechanisms including the roles of superoxide dismutase (SOD) and catalase (CAT) in protection and restoration of normal cellular homeostasis under oxidative stress [7]. Furthermore, nitric oxide (NO), a versatile and ubiquitous signaling molecule, is known to play diverse physiological functions in biological systems including cellular homeostasis, neurotransmission, cytoprotection, inflammation, host-defense responses [8,9], and is also known to provide some protection to the cellular system against the oxidative stress [10].

The facultative freshwater air-breathing magur catfish (*Clarias magur*), found predominantly in tropical southeast Asia, usually live in stagnant and polluted water bodies and are likely to get exposed to NPs, including the ZnO NPs, due to an infestation of industrial wastes and also due to anthropogenic activities. This group of catfish is reported to be more resistant to various environmental challenges such as high environmental ammonia, hypoxic and desiccation stresses [11]. Regardless of previous studies, the adaptational strategies against the ZnO NP-induced stressors in this catfish and other teleosts have not been well studied. Thus, the present study was aimed at determining the

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direct consequences of ZnO NP exposure on the cellular physiology in the context of oxidative imbalance and the possible cytoprotective role of NO, generated due to induction of inducible nitric oxide synthase (*nos2*) gene, against the NP-induced oxidative stress in isolated hepatocytes of magur catfish.

2. Materials and methods

2.1. Antibodies and reagents

A polyclonal antibody specific to iNOS was produced against the peptide from the epitope EIGARDFCDPQRYNILEKVGR. The peptide was conjugated to the KLH (Keyhole Limpet Hemocyanin) peptide to immunize the rabbit for obtaining the polyclonal antibody (Imagenex, India). Rabbit polyclonal NFκB p65 (sc-109), goat polyclonal CAT (sc-34285) antibody, goat polyclonal GAPDH (sc-48166), the HRP-conjugated anti-rabbit (sc-2357), anti-mouse (sc-2005) and anti-goat (sc-2020) IgG, Bay 11-7085 (BAY) and aminoguanidine (AG) were procured from Santa Cruz Biotechnology, USA. Rabbit polyclonal SOD (S4946) was procured from Merck, USA. Oligonucleotide primers were procured from GCC Biotech, India. SYBR® Premix Ex Taq™ II was procured from Takara, Japan. Other chemicals were of analytical grade and obtained from local sources. MilliQ water was used in all preparations.

2.2. Particle characterization

The ZnO NPs (544906) were purchased from Sigma-Aldrich (USA). The size and morphological structure of ZnO NPs were analyzed using a transmission electron microscope (TEM, JOEL, JEM-2100) with 200 kV operating voltage. The particle size distribution was determined using ImageJ software.

2.3. Hepatocyte isolation, culture and experimentation with ZnO NPs with or without inhibitors

Hepatocytes were isolated and cultured following the method of Banerjee et al. [12]. Hepatocyte viability of 80–90% with 5 and 10 μg/mL of ZnO NPs till 72 h was confirmed by MTT assay following the method of Mosmann [13]. A total of three sets of experiments were performed with two different concentrations of ZnO NPs (5 and 10 μg/mL) in the culture media. In the first set, hepatocytes were treated with ZnO NPs (5 or 10 μg/mL) alone, in set 2, with ZnO NPs (5 or 10 μg/mL) plus AG (3 μM) and in set 3, with ZnO NPs (5 or10 μg/mL) plus BAY (3 μM) for a period of 48 h.

2.4. Estimation

The intracellular hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) concentrations in primary hepatocytes were determined using Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher, USA) and TBARS assay kit (DTBA-100, BioAssay Systems, USA), respectively, following manufacturers' instructions. The NO concentration in the culture media and cell lysates were measured by treating both the culture media and cell lysates with 5% perchloric acid (PCA) in 1:0.5 ratio to precipitate out the proteins, followed by centrifugation at 10,000 × *g* for 5 min. The concentration of NO in the supernatant was determined following the Griess reaction as described by Sessa et al. [14]. In brief, to 1 mL of supernatant, 1 mL of Griess reagent was added and incubated at room temperature for 15 min. The purple azo product so developed was measured at 540 nm with a UV-visible spectrophotometer (Cary 60, Agilent, USA). A standard curve was prepared with sodium nitrite to calculate the NO concentration.

The protein concentration in cell lysates was determined using Bradford reagent [15].

2.5. Enzyme assay

The hepatocytes were ultrasonically lysed for 10 × 4 s in a lysis buffer containing 50 mM Tris-HCl (pH-7.4), 150 mM NaCl and protease inhibitor on the ice and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatants were used for assaying the activities of different enzymes.

The SOD activity was determined following the method of Paoletti et al. [16]. The reaction mixture in a final volume of 1 mL contained 50 mM potassium phosphate buffer (pH 7.5), 0.5 mM riboflavin and 0.4 mM nitroblue tetrazolium which was pre-incubated at 27 °C for 5 min in a quartz cuvette, followed by the addition of 10 μL of cell lysate to start the reaction. The formation of formazan blue was continuously monitored in a UV-visible spectrophotometer (Cary 60, Agilent) at 560 nm for 3 min at 27 °C. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT photoreduction.

The CAT activity was determined following the method of Beers and Sizer [17]. The reaction mixture in a final volume of 3 mL contained 50 mM potassium phosphate buffer (pH 7.5) and 25 mM H_2O_2, which was pre-incubated at 27 °C for 5 min in a quartz cuvette, followed by the addition of 10 μL of cell lysate to the reaction mixture to start the reaction. The H_2O_2 decomposition was continuously monitored in a UV-visible spectrophotometer (Cary 60, Agilent) at 240 nm for 3 min at 27 °C. One unit of CAT activity was defined as the amount of enzyme that decomposed 1 μmole of H_2O_2 per min at 27 °C. Both the SOD and CAT activities were expressed as units/mg of protein.

The iNOS activity was assayed following the method of Knowles and Salter [18] with certain modifications [19]. In one set of assay mixture, in a final volume of 1 mL contained 100 mM Na-phosphate buffer (pH 7.2), 100 mM L-arginine, 1.5 mM $MgCl_2$, 0.25 mM $CaCl_2$, 0.12 mM NADPH, 50 mM L-valine, 10 units of urease and 0.1 mL of cell lysate. The second set of assay mixture contained all the above reagents except $CaCl_2$, and also contained 0.5 mM aminoguanidine (AG) as a specific inhibitor of iNOS and 1 mM EGTA. Both sets of reaction mixtures were incubated at 27 °C for 20 min, and the reaction was stopped by adding 1 mL 10% PCA to precipitate out the protein, followed by centrifugation at 10,000 × *g* for 5 min. Citrulline, so formed as the reaction product, was estimated in the supernatant spectrophotometrically at 490 nm in a UV-visible spectrophotometer (Cary 60, Agilent) following the method of Moore and Kauffman [20] and expressed as enzyme activity. The part of the activity, which was inhibited in the second set of the reaction mixture, was taken as iNOS activity. One unit of enzyme activity was defined as that amount of enzyme which catalyzed the formation of 1 μmole of citrulline per h at 27 °C and expressed as units/mg of protein.

The lactate dehydrogenase (LDH) activity in the culture media was assayed spectrophotometrically following the method of Vorhaben and Campbell [21]. One unit of LDH activity was defined as that amount of enzyme which oxidized 1 μmol of NADH to NAD^+ per h at 27 °C and expressed as units/L.

2.6. Total RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR) analysis

The total RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis of mRNA expression of different genes were performed following Banerjee et al. [12]. The used primer pairs were designed, and the specificity of each primer pair was checked by using Primer-BLAST [22] (Table 1).

2.7. Western blot analysis

Western blot analysis of SOD, CAT, iNOS and NFκB p65 proteins were carried out following Banerjee et al. [12] by using a specific antibody against each protein. GAPDH was used as a protein loading control.

Table 1
Primer sets used in the study.

Primers	Forward (5′ - 3′)	Reverse (5′ - 3′)	Accession No
sod1	AGAGTGAGGATGCTCCCGTA	GCACTGATGCACCCATTTG	KF444052
sod2	TCAGCGTGACTTTGGCTCAT	GCCTCCCGTTCTCCTTATCG	MH891501
cat	GCTACGGATCCCACACCTTC	GTCACGGAGGGCATAGTCAG	KF977829
nos2	GGATGGACCCCAAAGTACGG	TGGGTGCTCCATTTCAACCT	KT180212
rela	ATTCCTGAAGCCAAGGTGTGG	AGAGGATACAGCTCCCCGTT	MG571500
βactin	GCCTCTTCCTCCTCTCTGGA	AGATGGCTGGAAGAGAGCCT	EU527190
tuba1	GTCGAGCAAGGTAACGAACTTCAC	GTGGATGGAGATACACTCACGCA	AB923912

2.8. Statistical analysis

The data, collected from different experiments, were statistically analyzed by One-way ANOVA test, followed by Tukey's multiple comparison test, performed in GraphPad Prism software and presented as mean ± S.E.M (n = 5 in each set of the experiment). Differences with $P < 0.05$ were regarded as statistically significant.

3. Results

3.1. Characterization of ZnO NPs

ZnO NPs were characterized by a transmission electron microscope. The average diameter of ZnO NPs was found to be about 45 nm (Fig. 1A), and the histogram of the size distribution of ZnO NPs is shown in Fig. 1B.

3.2. Intracellular H_2O_2 and MDA concentrations in hepatocytes

Exposure of hepatocytes to ZnO NPs at two different concentrations (5 and 10 μg/mL) led to a sharp rise of intracellular H_2O_2 concentration by 2.8- and 3.5-fold, respectively, within 6 h, followed by a gradual decrease at later stages, but remained significantly higher than the respective controls in both the concentrations of NPs after 48 h of treatment (Fig. 2A). Treatment of hepatocytes with 5 μg/mL NPs in the presence of AG and BAY separately, led to further enhancement of intracellular H_2O_2 concentration in hepatocytes by 4.1- and 5.3-fold, respectively, after 48 h (Fig. 2A). Likewise, the intracellular H_2O_2 concentration in hepatocytes also enhanced by 4.8- and 6-fold when treated with 10 μg/mL NPs in the presence of AG and BAY, respectively, after 48 h.

Similarly, the intracellular concentration of MDA also increased significantly in hepatocytes due to treatment with both low and high concentrations of ZnO (5 and 10 μg/mL) by 5.8- and 7-fold, respectively, within 6 h, which gradually decreased at later stages (Fig. 2B). At 5 μg/mL ZnO NPs concentration in the presence of AG and BAY separately led to the enhancement of MDA level by 8.4- and 8.5-fold, respectively, after 48 h (Fig. 2B). Likewise, the MDA level also enhanced further by 9.6- and 8.2-fold at 10 μg/mL concentration in the presence of AG and BAY, respectively after 48 h.

3.3. LDH leakage in the culture media

The LDH leakage from the primary hepatocytes was assayed as a measure of cellular damage caused by ZnO NPs and different inhibitors (Fig. 2C). After exposure to both the concentrations of ZnO NPs, the LDH leakage from hepatocytes increased gradually in the culture media, as evidenced by an increase in LDH activity to 6.51 and 7.69 units/L, respectively, at a low and high concentration of ZnO NPs after 48 h. However, when the hepatocytes were treated with ZnO NPs along with AG and BAY, the LDH leakage increased by many folds compared to the level obtained with ZnO NPs alone. The LDH activity in the culture media increased maximally to 45 units/L while treating the hepatocytes with 5 μg/mL ZnO NPs along with AG and BAY separately. Whereas, when the hepatocytes were treated with a high concentration of ZnO NPs (10 μg/mL) along with AG and BAY separately, the LDH leakage from primary hepatocytes increased further with a maximum activity of 60.4 and 55.6 units/L, respectively, after 48 h.

3.4. The SOD and CAT activities in hepatocytes

ZnO NPs treatment at a low concentration led to a significant increase of activity of SOD by 2.6-fold after 24 h, and 2.7-fold in case of CAT after 48 h in primary hepatocytes with an initial decrease of activities of both the enzymes within 6 h (Fig. 3A and D). Similarly, treatment with a high concentration of ZnO NPs led to a significant

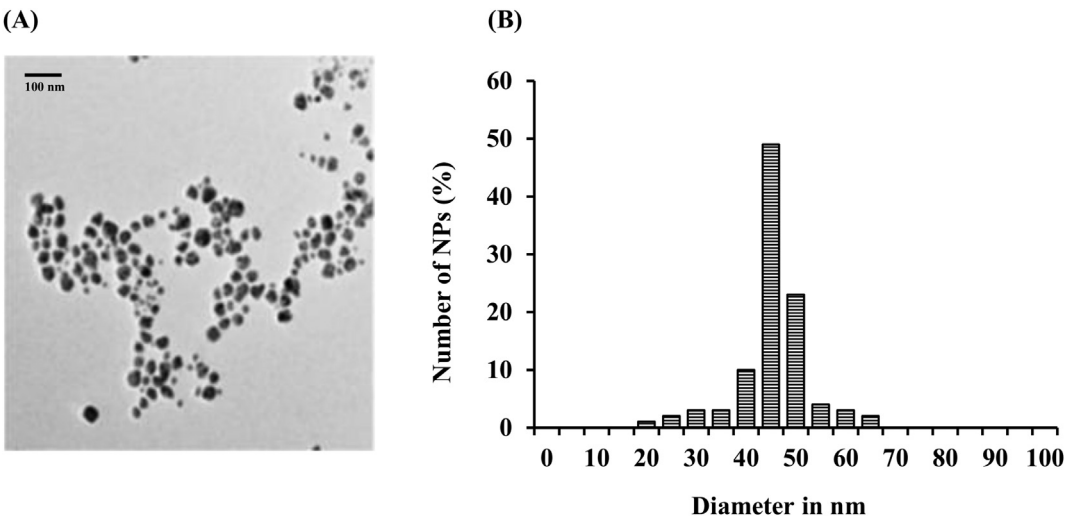

Fig. 1. (A) TEM micrograph of ZnO NPs and (B) the histogram of size distribution.

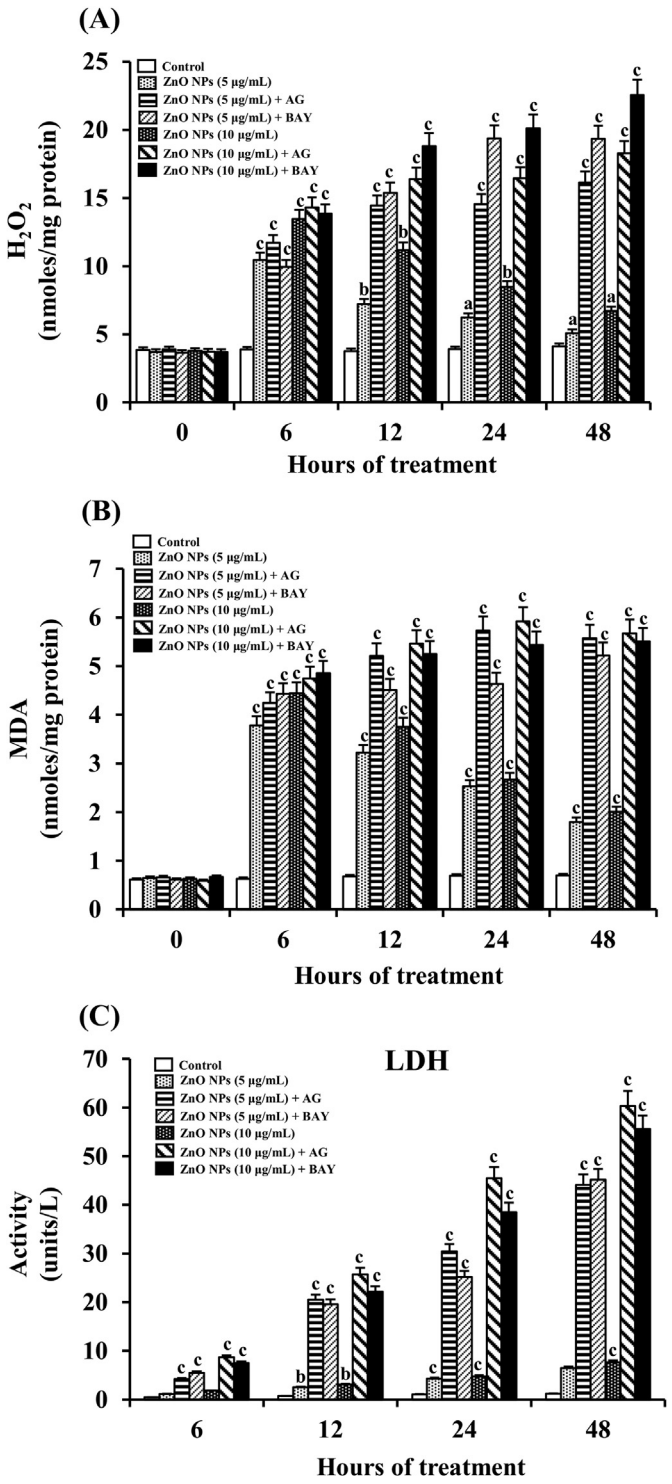

Fig. 2. Changes in the intracellular concentrations of (A) H_2O_2 (nmoles/mg of protein), (B) MDA (nmoles/mg of protein) in primary hepatocytes and (C) changes in the activity of LDH (units/L) in the culture media while treating with different concentrations of ZnO NPs in absence and presence of different inhibitors. ^{a,b,c}: *P* values significant at < 0.01, < 0.05 and < 0.001 levels, respectively, compared to respective controls (one-way ANOVA).

increase of activity of SOD by 3.1-fold after 24 h, and 3-fold in case of CAT after 48 h in primary hepatocytes with an initial decrease of activities of both the enzymes within 6 h (Fig. 3A and D).

However, the activities of both the enzymes decreased significantly in primary hepatocytes during treatment with both the concentrations of ZnO NPs when AG and BAY were added separately in the culture media.

3.5. Expression of mRNAs for sod1, sod2, and cat in hepatocytes

Treatment of primary hepatocytes with ZnO NPs led to a maximum increase in the expression of *sod1* mRNA by 2.5- and 3.1-fold, and for *sod2* mRNA, it increased by 2.6- and 3.4-fold at a concentration of 5 and 10 μg/mL respectively, after 48 h. Whereas, when the primary hepatocytes were treated with both the concentrations of ZnO NPs in the presence of AG and BAY separately, the mRNA levels for both *sod1* and *sod2* decreased gradually with increasing time of exposure (Fig. 3B and C). Although the expression of *cat* mRNA level in hepatocytes initially decreased within 6 h of treatment with both the concentrations of ZnO NPs, it gradually increased at later stages (Fig. 3E). Whereas, when the primary hepatocytes were treated with AG and BAY separately in the presence of both the concentrations of NPs, the *cat* mRNA level decreased significantly and remained at a lower level compared to control values till 48 h of treatment.

3.6. SOD and CAT enzyme proteins expression in hepatocytes

The level of SOD enzyme protein concentration increased maximally by 3.8- and 4-fold after 24 h, and in case of CAT enzyme protein by 3- and 3.6-fold after 48 h of exposure to 5 and 10 μg/mL of ZnO NPs, respectively, whereas both SOD and CAT expression decreased significantly with the addition of AG and BAY separately along with ZnO NPs (Fig. 4A, B & C).

3.7. NO concentration in the culture media and hepatocytes

Treatment of hepatocytes with ZnO NPs led to a gradual increase of NO concentration in the culture media with a maximum increase of 5.9- and 8.2-fold at low and high concentrations of ZnO NPs, respectively, after 48 h. However, this increase of NO concentration was restricted only to about 2.5-fold when AG and BAY were added separately along with both the concentrations of ZnO NPs (Fig. 5A). Similarly, the intracellular concentration of NO also increased significantly due to treatment with low and high concentrations ZnO NPs, resulting in a maximum increase of 5.9- and 6.6-fold, respectively, after 48 h. Nonetheless, when AG and BAY were added separately in the culture media along with both the concentrations of ZnO NPs, the NO concentration increased only by 2.25-fold (Fig. 5B).

3.8. The iNOS activity, expression of nos2 and rela mRNAs and their translated products

A very negligible amount of iNOS activity could be detected in control hepatocytes. However, when the hepatocytes were treated with ZnO NPs, the iNOS activity increased significantly within 6 h, followed by a further increase at later stages of treatment with a maximum increase at a higher concentration of ZnO NPs after 48 h (Fig. 5C). However, when BAY was added in the culture media along with ZnO NPs, a negligible increase of iNOS activity could be detected in both the concentrations of ZnO NPs.

Treatment of primary hepatocytes with ZnO NPs led to a maximum increase of expression of *nos2* mRNA by 11.5- and 20.1-fold, respectively, at low and high concentrations, after 48 h. However, this increase in *nos2* mRNA level was confined only to about 4.3- and 2.6-fold, respectively, after 48 h at low and high concentrations of ZnO NPs in the presence of BAY in the culture media (Fig. 5D).

The iNOS enzyme protein level increased maximally by 6.1- and 9-fold after 48 h of exposure to 5 and 10 μg/mL of ZnO NPs, respectively. Whereas, in the presence of BAY along with both concentrations of ZnO NPs, the iNOS protein expression increased only by 2.2-fold after 48 h (Fig. 6A and C).

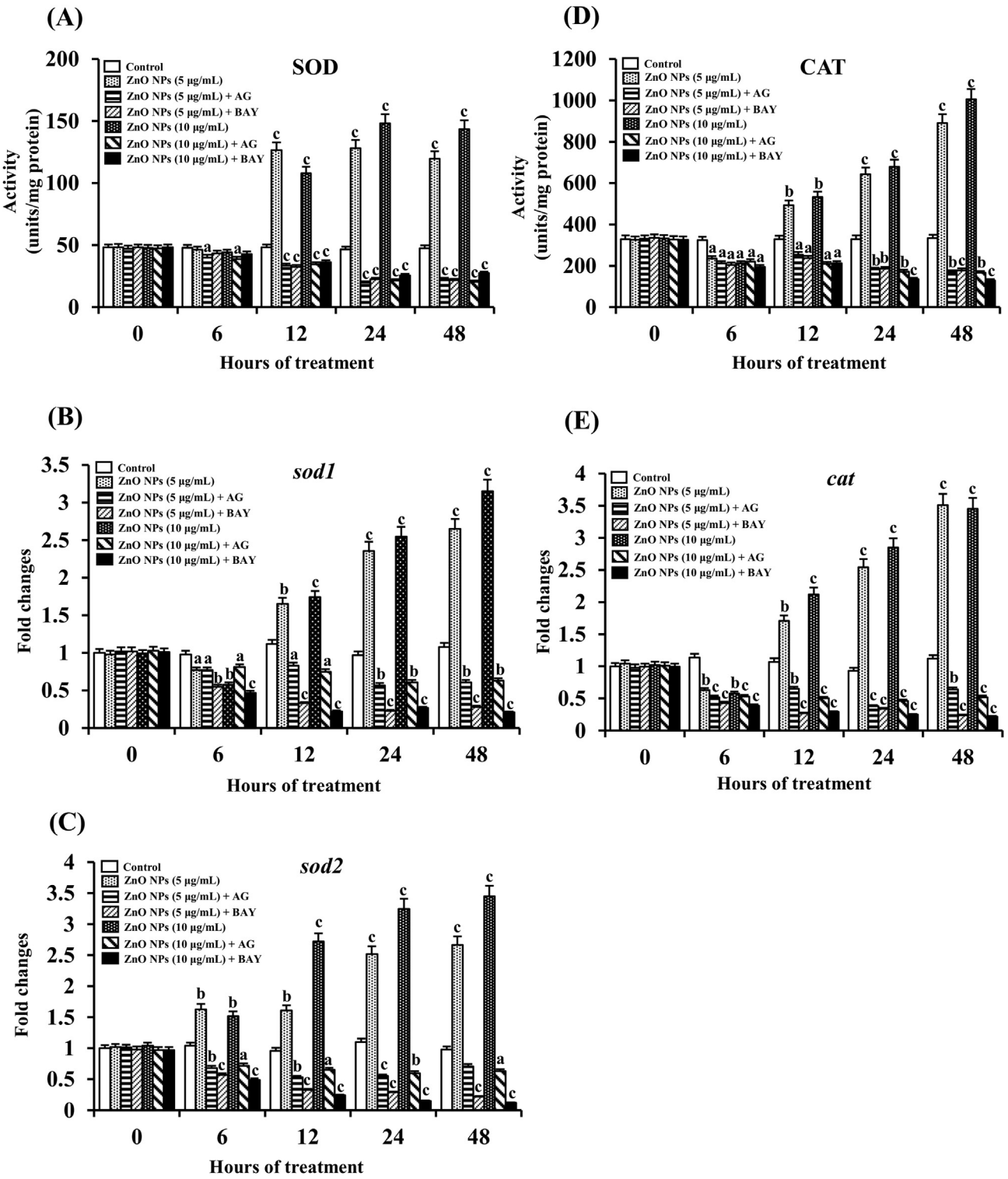

Fig. 3. Changes of activities of (A) SOD (units/mg of protein), fold changes in the expression of mRNAs for (B) *sod1*, (C) *sod2*, (D) changes of activities of CAT (units/mg of protein), and (E) fold changes in the expression of mRNAs for *cat* in primary hepatocytes of *C. magur* during exposure to ZnO NPs (5 and 10 µg/mL) in absence and presence of different inhibitors at different time intervals. Values are expressed as mean ± SEM (n = 5). ^{a,b,c}: *P* values significant at < 0.01, < 0.05 and < 0.001 levels, respectively, compared to respective controls (one-way ANOVA).

ZnO NPs treatment also led to a significant increase in the expression of *rela* mRNA with a maximum increase of 3.7- and 4.1-fold, respectively, at low and high concentrations of ZnO NPs after 48 h (Fig. 5E), This was accompanied by a significant increase in the level of NFκB p65 protein by 3.2- and 3.6-fold, respectively, at low and high concentrations of ZnO NPs (Fig. 6B and D).

4. Discussion

The results of the present study demonstrated that treatment of primary hepatocytes of magur catfish with both low (5 µg/mL) and relatively high (10 µg/mL) concentrations of ZnO NPs in the culture media initially evoked generation of more ROS. The elevated level of

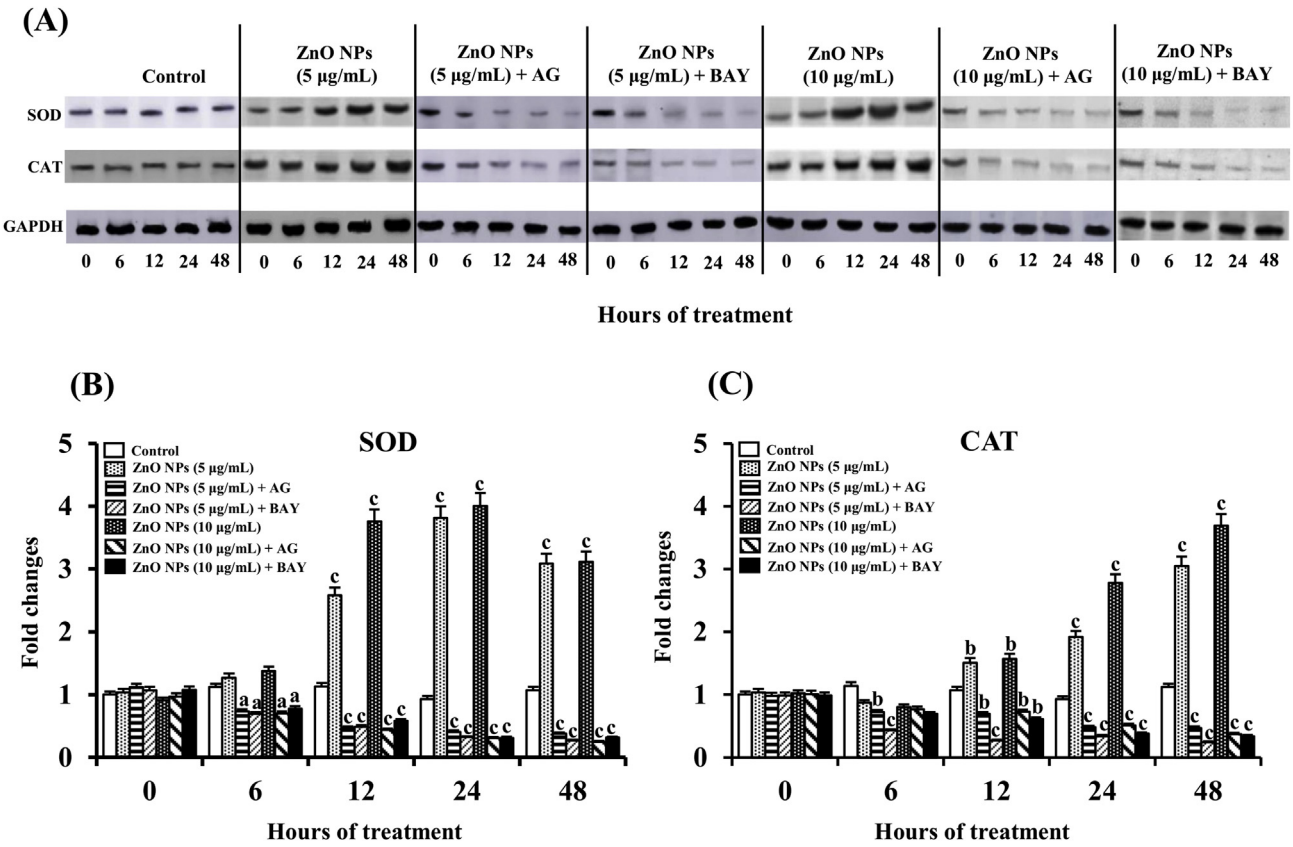

Fig. 4. (A) Western blot analysis showing changes in the levels of expression of SOD and CAT proteins in primary hepatocytes of *C. magur* during exposure to ZnO NPs (5 and10 µg/mL) in the absence and presence of different inhibitors at different time intervals. Densitometric analysis showing fold changes of (B) SOD and (C) CAT protein concentrations compared to respective controls in primary hepatocytes at different time intervals. Values are expressed as mean ± SEM (n = 5). ^{a,b,c}: *P* values significant at < 0.01, < 0.05 and < 0.001 levels, respectively, compared to respective controls (one-way ANOVA).

ROS ultimately caused oxidative stress as evidenced by a sharp rise of intracellular H_2O_2 and MDA concentrations within 6 h, but at later stages, both decreased gradually. Similarly, the activities of two antioxidant enzymes, SOD and CAT also decreased initially, followed by a subsequent increase at later stages. Parallel to these changes in the activities of these two antioxidant enzymes, the expression of *sod1*, *sod2* and *cat* genes, and their translated products also decreased initially, but subsequently increased significantly at later stages. Thus, it appears that the primary hepatocytes can defend themselves against the ZnO NP-induced oxidative stress by inducing the *sod* and *cat* genes as the first line of defense. However, it is to be noted that NP-induced oxidative stress in primary hepatocytes worked in a dose-dependent manner since there was less generation of both H_2O_2 and MDA at a lower dose of ZnO NPs than at a higher dose. Similarly, the antioxidant activity was found to be more responsive at a higher dose of ZnO NPs. Similar dose-dependent cytotoxic effect of ZnO NPs was also reported in the human immune cell (THP-1) [23] and rat retinal ganglion cell (RGC-5) [24]. ZnO NP-induced oxidative stress was reported in primary hepatocytes of channel catfish (*I. punctatus*) [25], but unlike that of magur catfish, the presence of an antioxidant strategy in channel catfish hepatocytes was not reported.

The most important observation that has been made in the present study is the induction of NOS/NO synthetic machinery in our fish hepatocytes under ZnO NPs-induced oxidative stress. ZnO NP treatment led to higher production of NO resulting in a significant increase of NO concentration in primary hepatocytes and in the culture media, which was accompanied by stimulation of iNOS activity in primary hepatocytes. It probably resulted due to the stimulation of *nos2* gene and iNOS enzyme protein levels in the primary hepatocytes. Furthermore, similar to the antioxidant enzyme activities, the level of induction of *nos2* gene and NO production by the primary hepatocytes, were found to be dose-dependent. Similarly, NO-mediated antioxidant mechanisms were also suggested in yeast (*Saccharomyces cerevisiae*) [26] and fission yeast (*Schizosaccharomyces pombe*) [27]. Nasuno et al. [26] proposed that NO activate the transcription factor MAC1, which ultimately induces the *ctr1* gene leading to enhancement of cellular copper (Cu) level, which ultimately activates SOD1 activity. Recent studies indicated that NO concentration contributes towards more tolerance to H_2O_2 by inhibiting the Fe^{3+} to Fe^{2+} conversion and by upregulating the H_2O_2 detoxifying enzymes [9,27]. Furthermore, Husain et al. [28] suggested the NO-mediated inhibition of electron transport chain in *Salmonella* as a novel antioxidant strategy. Likewise, Robb and Connor [29] also reported the protective role of NO against the oxidative stress-induced mitochondrial damages of astrocytes. However, it is to be noted that in all these studies, either the substrates for NO or the NO itself was added directly in the experimental system to check the antioxidant activity of NO. Though, in our study, neither NO nor any substrate for NO production was added in the culture media to investigate the antioxidant activity of NO under ZnO NP-induced oxidative stress. Instead, we observed that more production of endogenous NO in the primary hepatocytes was mediated through the induction of *nos2* gene during exposure to ZnO NPs.

Interestingly, when the primary hepatocytes were treated with AG along with both the concentrations of ZnO NPs, the intracellular concentrations of both H_2O_2 and MDA increased prominently compared to the levels that were observed in the presence of NPs alone with a significant decrease of NO concentration. This increase of H_2O_2 and MDA levels were accompanied by a significant decrease of SOD and CAT activities along with the inhibition of expression of mRNAs and enzyme proteins. Similar observations were also made when the primary

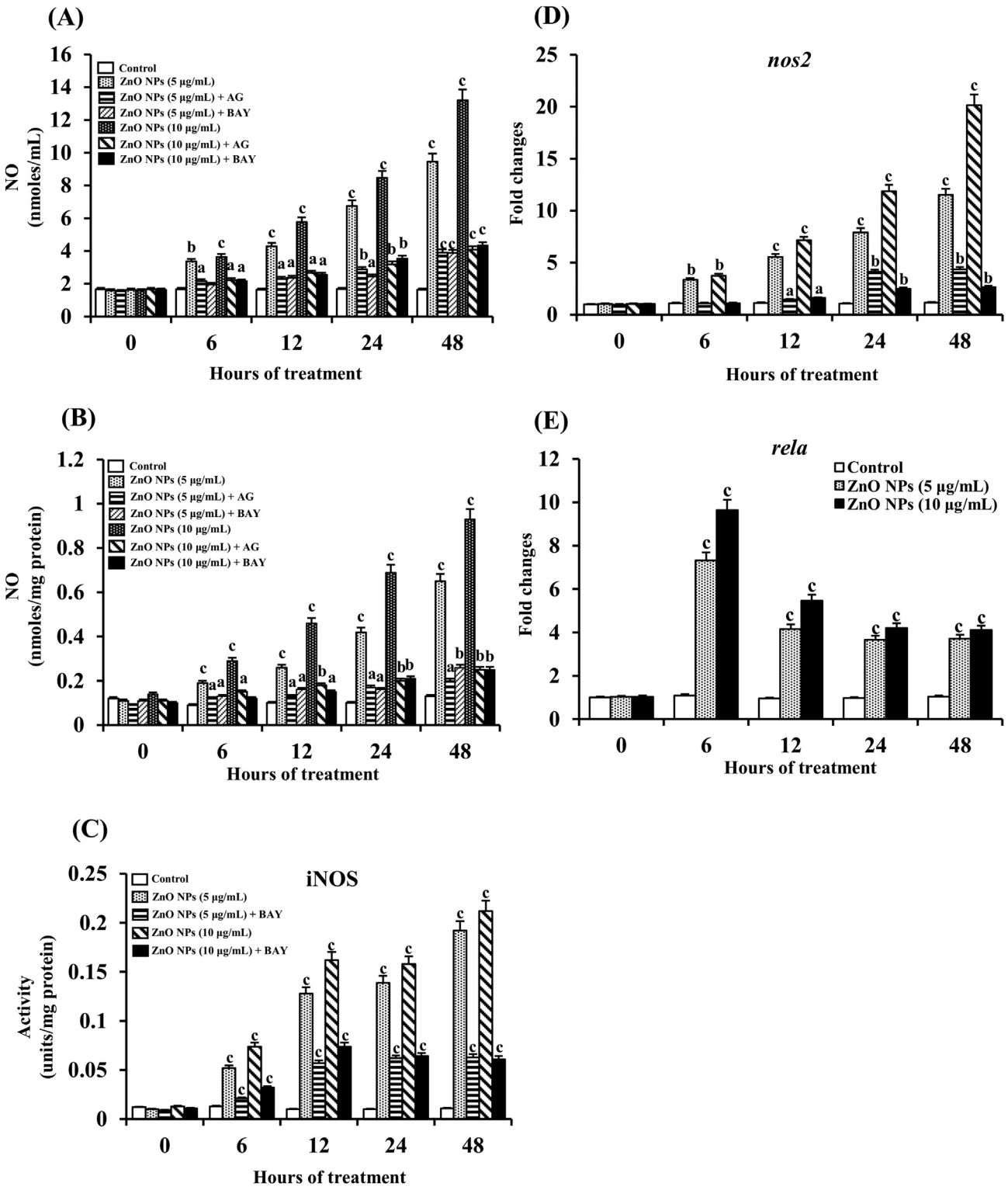

Fig. 5. Changes in the level of (A) NO in the culture media (nmoles/mL), (B) intracellular concentration of NO (nmoles/mg protein), (C) changes of iNOS activity (units/mg of protein), (D) fold changes in the expression of *nos2* mRNA and (E) fold changes in the expression of *rela* mRNA in primary hepatocytes of *C. magur* during exposure to ZnO NPs (5 and 10 µg/mL) in the absence and presence of different inhibitors. Values are expressed as mean ± SEM (n = 5). ^{a,b,c}: *P* values significant at < 0.01, < 0.05 and < 0.001 levels, respectively, compared to respective controls (one-way ANOVA).

hepatocytes were treated with BAY in the presence of ZnO NPs. Additionally, it is interesting to note that significant elevation of intracellular concentrations of H_2O_2 and MDA, due to inhibition of iNOS mediated NO production, was accompanied by more of LDH leakage from the primary hepatocytes to the culture media as a consequence of cellular damages under oxidative stress.

Furthermore, ZnO NPs treatment led to a significant induction of both *rela* gene and NFκB (p65) protein levels. More recently, the NFκB-mediated induction of *nos2* gene has been reported in another closely related air-breathing stinging catfish (*Heteropneustes fossilis*) under

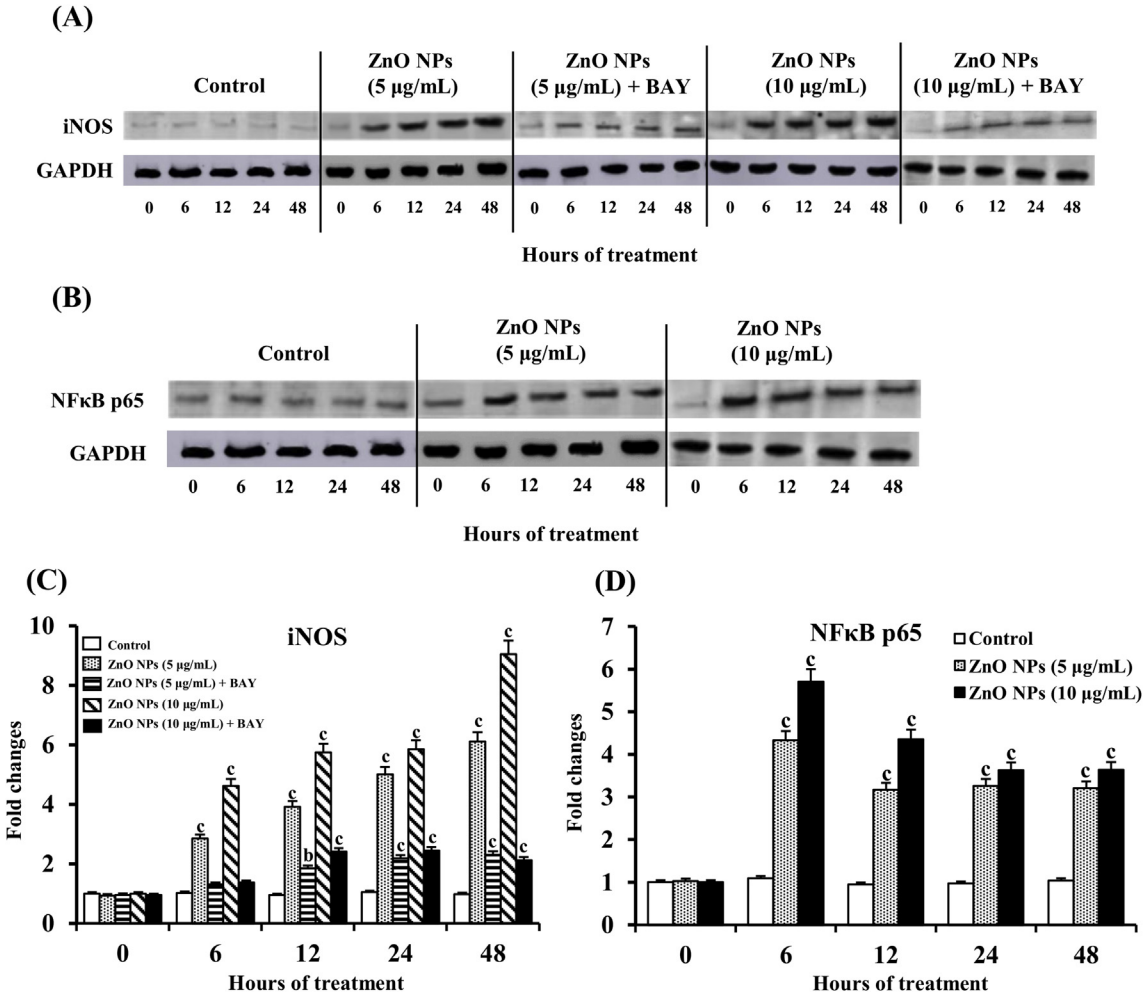

Fig. 6. Western blot analysis showing changes in the levels of expression of (A) iNOS and (B) NFκB p65 proteins in primary hepatocytes of *C. magur* during exposure to ZnO NPs (5 and10 µg/mL) in the absence and presence of different inhibitors at different time intervals. Densitometric analysis showing fold changes of (C) iNOS and (D) NFκB p65 protein concentrations compared to respective controls at different time intervals. Values are expressed as mean ± SEM (n = 5). ^{a,b,c}: *P* values significant at < 0.01, < 0.05 and < 0.001 levels, respectively, compared to respective controls (one-way ANOVA).

hyper-ammonia stress [19]. Thus, it is very much logical to think that the induction of *nos2* gene, which took place as an antioxidant strategy in the primary hepatocytes of magur catfish under ZnO NP treatment, was also mediated through NFκB.

Thus, from all these observations made in the present study, it is very much evident that the hepatocytes of magur catfish can reduce the NP-induced oxidative stress by inducing the SOD/CAT enzyme system mediated by NO generated endogenously, due to the induction of *nos2* gene. It is the first report of simultaneous induction of NO-producing system and SOD/CAT enzyme system as antioxidant strategies against the NP-induced oxidative stress in any teleost fish, as a self-defensive mechanism to shield against the oxidative stress-related cellular damages.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2018.12.010.

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