Free Radical Research, September 2011; 45(9): 1033–1039

informa
healthcare

Cisplatin induces production of reactive oxygen species via NADPH oxidase activation in human prostate cancer cells

TOMOHIRO ITOH[1,2], RIYAKO TERAZAWA[2], KEITARO KOJIMA[3], KEITA NAKANE[3], TAKASHI DEGUCHI[3], MASASHI ANDO[1], YASUYUKI TSUKAMASA[1], MASAFUMI ITO[2] & YOSHINORI NOZAWA[2,4]

[1]*Faculty of Agriculture, Kinki University, 3327-204 Nakamachi, Nara, 631-8505, Japan,* [2]*Gifu International Institute of Biotechnology, 1-1 Naka-Fudogaoka, Kakamigahara, Gifu, 504-0838, Japan,* [3]*Department of Urology, Gifu University Graduate School of Medicine, 1-1 yanagido, Gifu, Gifu 501-1193, Japan, and* [4]*Tokai Gakuin University, 5-68 Naka-Kirino, Kakamigahara, Gifu 504-8511, Japan*

(Received date: 25 January 2011; Accepted date: 22 May 2011)

Abstract

This study aimed to examine the roles of reactive oxygen species (ROS) in cisplatin treatment of human prostate cancer cells; hormone-sensitive LNCaP and hormone-refractory PC3 and DU145 cells. Intracellular levels of ROS and H_2O_2 were measured and visualized using specific fluorescent probes. NADPH oxidase (NOX) activity was detected by lucigenin chemiluminescence assay. Expression levels of NOX isoforms were determined by semi-quantitative RT-PCR. Cisplatin treatment increased the intracellular levels of ROS and H_2O_2 in three prostate cancer cell lines. The increase was transient and robust in hormone-sensitive LNCaP cells compared with hormone-refractory PC3 and DU145 cells. Consistent with these findings, the NOX activity induced by cisplatin was higher in LNCaP cells than in PC3 and DU145 cells. Expression pattern of NOX isoforms varied among three cell lines and the NOX activity was independent of NOX expression. Taken together, we have shown that cisplatin induces production of ROS and H_2O_2 via NOX activation in human prostate cancer cell lines, which is most prominent in hormone-sensitive LNCaP cells.

Keywords: *Prostate cancer cells, cisplatin, reactive oxygen species, NADPH oxidase, chemosensitivity*

Introduction

It has been reported that taxol induces oxidative neuronal cell death by enhancing the NOX activity in mouse cortical cultures [1]. On the other hand, many reports have indicated that inhibition of ROS with antioxidant and NOX inhibitors leads to cell death [2–4]. It has thus been suggested that ROS is an important regulatory factor for cell growth and survival [5,6]. In prostate cancer cells, ROS generated by NOX are essential for cell growth [7,8]. However, the relationship between ROS and anti-cancer drugs in prostate cancer cells has yet to be clarified. In this study, we investigated and found that ROS and H_2O_2 are produced in response to cisplatin treatment in human prostate cell lines and that NOX is involved in the cisplatin-induced production of ROS and H_2O_2.

Materials and methods

Reagents

Camptothecin, N-acetyl-L-cysteine (NAC), rotenone and diphenyliodonium (DPI) were from Sigma (St. Louis, MO). Cisplatin was obtained from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell culture and treatment

PrEC normal human prostate epithelial cells were obtained from Lonza (Basel, Switzerland, MD) and cultured as recommended by the supplier. LNCaP, PC3 and DU145 human prostate cancer cells were purchased from American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum. Cisplatin

Correspondence: Tomohiro Itoh, Gifu International Institute of Biotechnology, 1-1 Naka-Fudogaoka, Kakamigahara, Gifu 504-0838, Japan. Tel: 81-583-71-4646. Fax: 81-583-71-4412. Email: titoh@giib.or.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10715762.2011.591391

and camptothecin dissolved in DMSO were added to the cell culture medium, with the final concentration of DMSO not exceeding 0.3% that showed no significant effect on cell growth (data not shown). Viable cell number was measured by trypan blue dye exclusion method.

Measurement of intracellular ROS and H_2O_2 levels

Intracellular levels of ROS and H_2O_2 were measured using 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCF -DA, Invitrogen, Carlsbad, CA) and BES-H_2O_2 (Wako), respectively. We used CM-H_2DCF-DA to detect ROS including superoxide (O_2^{-}•), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO•) and BES-H_2O_2 to specifically detect H_2O_2. Cells were stained with 10 μM CM-H_2DCF-DA or 25 μM BES-H_2O_2 for 30 min at 37°C. After washing twice with PBS, cells were mounted on the slide glass. Then, photomicrographs were taken with a fluorescent microscope (Olympus BX-50, Tokyo, Japan). For quantitation of the intracellular ROS levels, cells stained with CM-H_2DCF-DA were lysed and fluorescence was measured at 490 nm for excitation and at 530 nm for emission using a fluorometer (MTP-600F, Corona Electric, Hitachinaka, Japan). The ROS level was expressed as an arbitrary unit.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured using a fluorescent dye, Mito-Tracker Red (Invitrogen) that accumulates selectively in active mitochondria and becomes fluorescent when oxidized. Cells were treated with Mito-Tracker Red (10 nM) for 30 min. After washing twice with PBS, cells were subjected to microscopic observation.

Measurement of NOX activity

Membrane fractions were prepared using a Proteo Extract Subcellular Proteome Extraction kit (Calbiochem). The NOX activity was measured according to the method of Li et al. [9] with a slight modification. Briefly, lucigenin chemiluminescence assay was performed in the presence of 5 μM lucigenin (Sigma) and 100 μM NADPH (Sigma) as a substrate. Reaction was initiated by adding 10 μl of membrane fractions. In the absence of NADPH, no enzymatic activity was detected. The chemiluminescence signal was collected for 10 min with a luminescence reader (TD-20/20, Promega, Madison, WI).

Reverse transcription-PCR

Total RNA was extracted from cells by TRIzol reagent (Invitrogen) followed by DNase I treatment. cDNA was synthesized from 0.5 μg of total RNA using the PrimeScript reagent kit (Takara Bio, Ohtsu, Japan). PCR primers and conditions are described in Table I. PCR products were analysed by electrophoresis on 3% agarose gels.

Statistical analysis

All data were analysed by one-way ANOVA with Fisher's-multiple comparison test. The differences among the means were considered significant at $p < 0.05$.

Table 1. Primer sequences and semi-quantitative RT-PCR condition for various NOX isoforms.

Primer sequence	Semi-RT-PCR condition	
NOX1 (5′→3′) Forward: AAACAGAGGAGAGCATGAATGAGAG Reverse: AAGAATGACCGGTGCAAGGA	40 cycle	Denaturation: 94°C for 30 sec Annealing: 60°C for 30 sec Extension: 72°C for 30 sec
NOX2 (5′→3′) Forward: TGGTGATGTTAGTGGGAGCA Reverse: GCCAGTGAGGTAGATGTTGTAGC	40 cycle	Denaturation: 94°C for 30 sec Annealing: 60°C for 30 sec Extension: 72°C for 30 sec
NOX3 (5′→3′) Forward: TCACAAACTGGTCGCCTATG Reverse: GTTGTGTTTGTGGGGAAGGT	40 cycle	Denaturation: 94°C for 30 sec Annealing: 60°C for 30 sec Extension: 72°C for 30 sec
NOX4 (5′→3′) Forward: TGAATGCAGCAAGATACCGAGA Reverse: TGAGGAATAGCACCACCACCA	55 cycle	Denaturation: 94°C for 30 sec Annealing: 65°C for 30 sec Extension: 72°C for 30 sec
NOX5 (5′→3′) Forward: CAACAAGGAGAAGAAAGACTCCAT Reverse: GTGAGGCTAGAAATTCTCTTGGAA	30 cycle	Denaturation: 94°C for 30 sec Annealing: 55°C for 30 sec Extension: 72°C for 30 sec
GAPDH (5′→3′) Forward: CCACCCATGGCAAATTCCATGGCA Reverse: TCTAGACGGCAGGTCAGGTCCACC	20 cycle	Denaturation: 94°C for 30 sec Annealing: 60°C for 30 sec Extension: 72°C for 30 sec

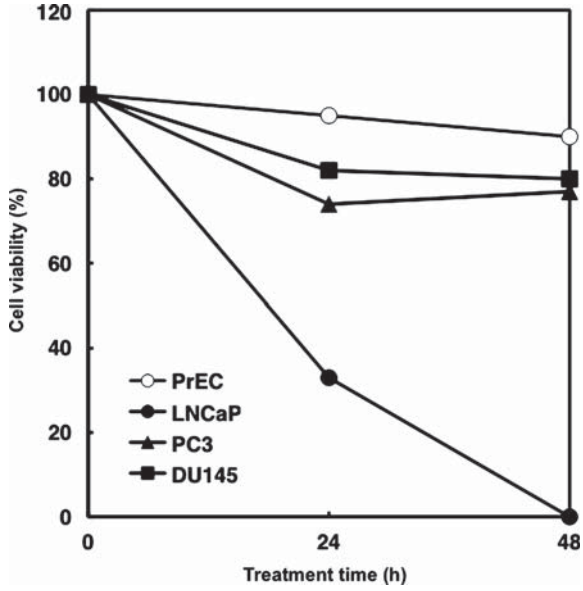

Figure 1. Effects of cisplatin on growth of PrEC, LNCaP, PC3 and DU145 cells. Each cell line was treated with 10 μM cisplatin for 48 h. Viable cell number was measured by the trypan blue dye exclusion method. ○: PrEC, ●: LNCaP, ▲: PC3, ■: DU145.

Results

Chemosensitivity of human prostate cancer cells to cisplatin

We examined cytotoxic effects of cisplatin on PrEC, LNCaP, PC3 and DU145 cells by trypan blue dye exclusion method. The viable cell number of LNCaP cells was remarkably decreased by cisplatin treatment, whereas that of PrEC was not affected and that of PC3 and DU145 cells were slightly decreased (Figure 1). These results indicate that LNCaP cells are more sensitive to cisplatin than PrEC, PC3 and DU145 cells.

Intracellular ROS generation by cisplatin treatment in human prostate cancer cells

We determined intracellular ROS levels using CM-H_2DCF-DA in order to examine the effect of cisplatin on ROS generation. As shown in Figure 2, the intracellular ROS levels in hormone-sensitive LNCaP cells robustly (1200%) and transiently (5–15 min) increased following cisplatin treatment and remained elevated thereafter. ROS generation was also seen in hormone-refractory PC3 and DU145 cells at 60 min after cisplatin treatment, but to a much lesser extent. In PrEC normal human prostate epithelial cells, however, little or no significant ROS production was observed in response to cisplatin. A similar profile of ROS generation was also seen with another anti-cancer drug, camptothecin, topoisomerase II inhibitor. Furthermore, we examined microscopically intracellular ROS and H_2O_2 production in cisplatin-treated PrEC, LNCaP and PC3 cells using ROS and H_2O_2-specific fluorescence probes. The data presented in Figure 3 show that production of ROS and H_2O_2 in LNCaP cells by cisplatin treatment was much higher than that in PC3 cells. Cisplatin-induced ROS and H_2O_2 production in DU145 cells was almost similar to that observed in PC3 cells (data not shown). On the other hand, mitochondrial membrane potential was not changed by cisplatin treatment in all prostate cell lines tested, suggesting that cisplatin-induced ROS production is unlikely due to defective mitochondria. Since NOX generates superoxide, which is immediately converted to H_2O_2, it was considered that H_2O_2 production in cisplatin-treated cells was a consequence of the conversion of superoxide generated by NOX.

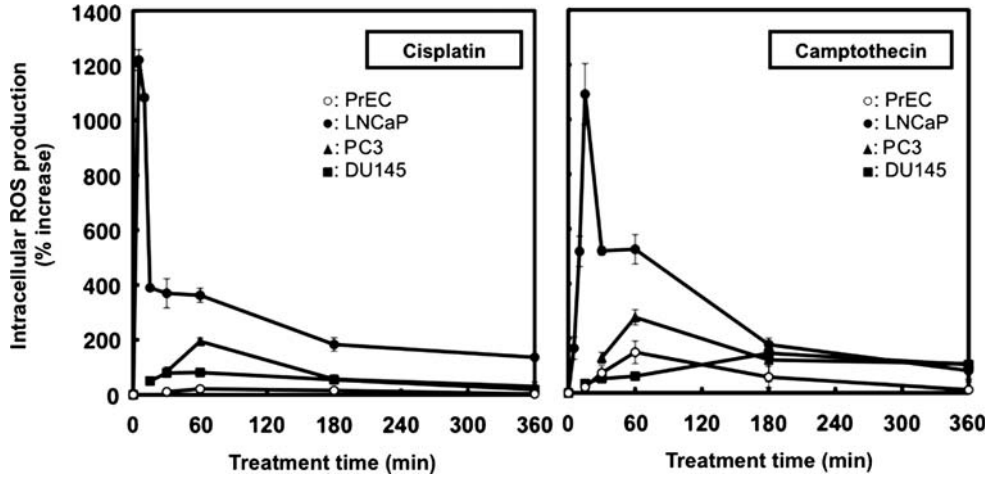

Figure 2. Effects of cisplatin and camptothecin treatment on intracellular ROS generation in PrEC, LNCaP, PC3 and DU145 cells. Intracellular ROS levels were determined using CM-H_2DCF-DA at indicated times after treatment with cisplatin (10 μM) or camptothecin (10 μM) in PrEC (○), LNCaP (●), PC3 (▲) and DU145 (■). Values are mean ± SEM ($n = 9$).

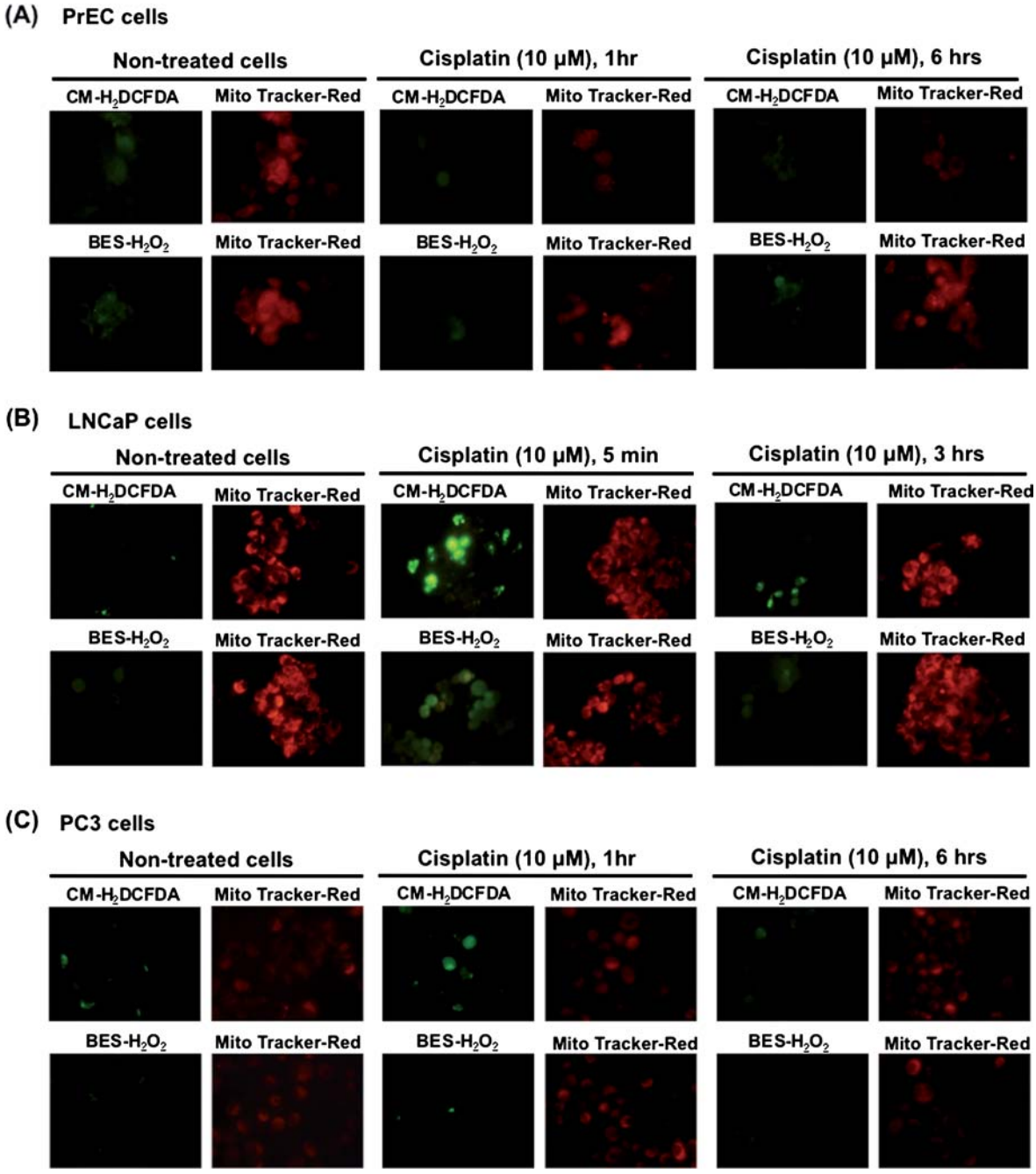

Figure 3. Effects of cisplatin treatment on intracellular ROS and H_2O_2 generation and mitochondrial membrane potential in PrEC, LNCaP and PC3 cells. Intracellular ROS and H_2O_2 in cisplatin-treated PrEC (A), LNCaP (B) and PC3 (C) cells were visualized using CM-H_2DCF-DA (for detecting ROS; H_2O_2, superoxide and hydroxyl radicals) and BES-H_2O_2 (for detecting H_2O_2). Mitochondrial membrane potential was visualized using Mito-Tracker Red.

Involvement of NOX in intracellular ROS generation by cisplatin treatment

To gain further insight into the mechanisms underlying cisplatin-induced ROS production, we investigated the effects on ROS production in cisplatin-treated cells of antioxidant, NAC (1 mM), mitochondrial complex I inhibitor, rotenone (10 μM) and NOX inhibitor, DPI (200 nM). Cisplatin-induced ROS generation in PC3 and DU145 cells was significantly suppressed by pre-treatment with NAC and DPI, whereas rotenone pre-treatment showed a marginal effect (Figure 4). We were unable to examine the effects of these agents on cisplatin-induced ROS generation in LNCaP cells, because each of them alone exhibited a cytotoxic effect (Supplementary Figure 1).

NOX activity and expression in human prostate cancer cells

The basal levels of NOX activity and ROS concentration in DU145 cells were lower than those of LNCaP and PC3 cells (Figure 5A). Cisplatin treatment increased

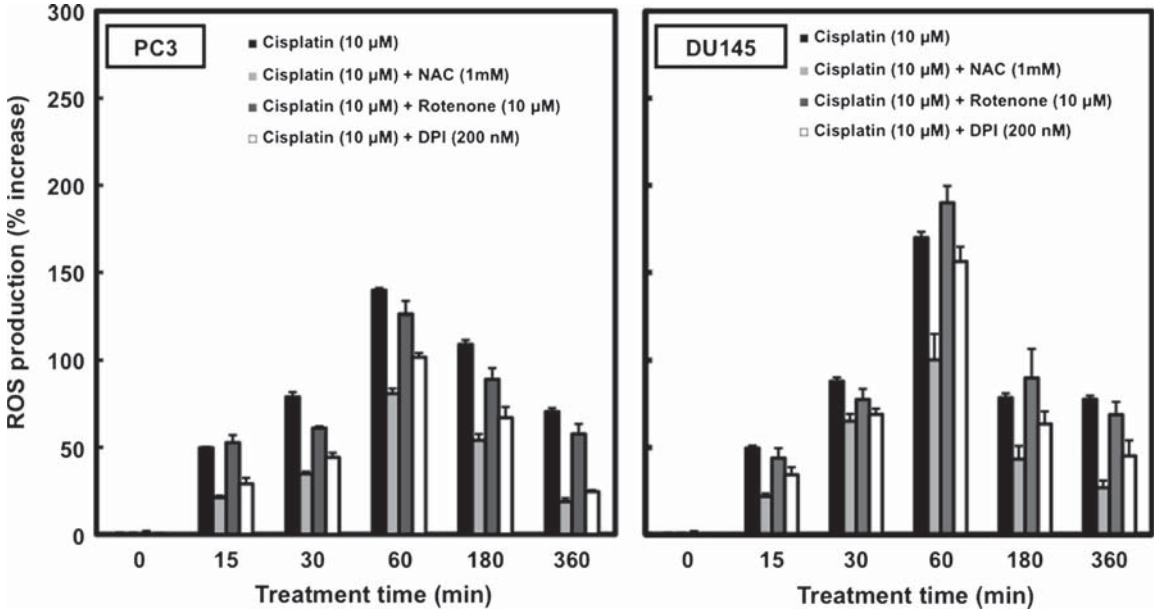

Figure 4. Effects of NAC, rotenone and DPI pre-treatment on cisplatin-induced ROS production in PC3 and DU145 cells. Cells were pre-treated with either NAC (1 mM), rotenone (10 μM) or DPI (200 nM) for 1 h at 37°C and then treated with cisplatin. Intracellular ROS levels were measured at indicated times using CM-H_2DCF-DA. Values are mean ± SEM ($n = 9$).

the NOX activity in LNCaP, PC3 and DU145 cells (Figure 5B). Cisplatin-induced NOX activation was higher in LNCaP cells than in PC3 and DU145 cells, which correlated with the differences in cisplatin-induced production of ROS and H_2O_2 among these cell lines (Figures 2 and 3).

We further determined the mRNA expression levels of *NOX* isoforms by semi-quantitative RT-PCR in LNCaP, PC3 and DU145 cells (Figure 5C). Expression of *NOX1*, *2* and *3* were observed in all these cell lines. *NOX2* was highly expressed in PC3 and DU145 cells, while *NOX5* was present only in PC3 cells and *NOX4* was not detected in any cell line.

Discussion

In the present study, we investigated the roles of ROS in anti-cancer drug treatment of human prostate cancer cell lines. It has been previously reported that several cancer cells produce ROS, which cause DNA damage and activation of nuclear factor κB (NF-κB), activator protein-1 and mitogen-activated protein (MAP) kinase and regulate cell migration and proliferation [10–13]. Indeed, intracellular ROS production was detected in human prostate cancer cell lines (Figure 5A). We further demonstrated that cisplatin and camptothecin transiently increase intracellular ROS levels in human prostate cancer cell lines (Figure 2). Other anti-cancer drugs have been also shown to generate ROS in several cancer cell lines [14–16]. It was thus postulated that ROS generated by cisplatin may damage proteins, lipids and DNA, as well as mitochondria, leading to cell death such as apoptosis [17,18]. Intriguingly, ROS production induced by cisplatin and camptothecin was much higher in LNCaP cells than in PC3 and DU145 cells (Figure 2). It was also found that LNCaP cells are more sensitive to cisplatin than PrEC, PC3 and DU145 cells (Figure 1) [19]. These findings suggest that a robust increase in ROS production may explain chemosensitivity of LNCaP cells to cisplatin. In contrast, PC3 and DU145 cells were resistant to cisplatin treatment (Figure 1). It has been reported that docetaxel-treated breast cancer cells up-regulate expression of redox-related genes such as thioredoxin, glutathione S-transferase and peroxiredoxins [20]. It is therefore possible that high adaptability to oxidative stress of PC3 and DU145 cells attenuated excess ROS production that would lead to cell death.

To clarify the mechanisms underlying ROS production by cisplatin treatment, we visualized intracellular ROS and H_2O_2 production in response to cisplatin treatment. We found that cisplatin-induced production of ROS and H_2O_2 in LNCaP cells was much higher than that in PC3 cells (Figure 3). We also examined the effects of NAC, rotenone and DPI on intracellular ROS production in cisplatin-treated cells. ROS production by cisplatin in PC3 and DU145 cells were significantly suppressed by pre-treatment with NAC and DPI, but not with rotenone (Figure 4). These results suggested that production of ROS by cisplatin is likely due to activation of NOX. However, we could not examine the mechanism of cisplatin-induced ROS generation in LNCaP cells, because the same treatments caused their cell death. Several

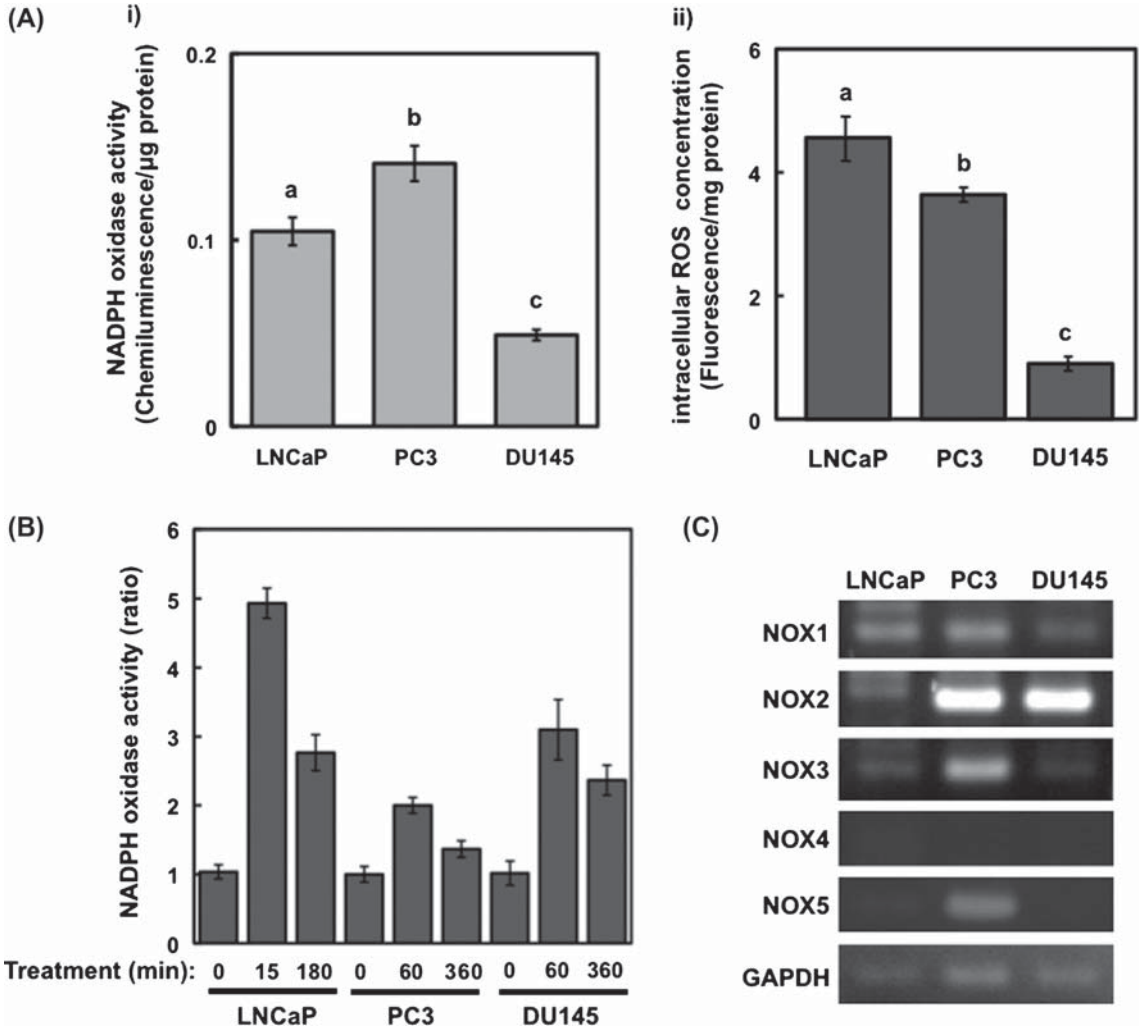

Figure 5. Effects of cisplatin treatment on NOX activity and expression in LNCaP, PC3 and DU145 cells. (A) The basal levels of NOX activity (i) and intracellular ROS levels (ii) in LNCaP, PC3, and DU145 cells. Means values with different letters are significantly different ($p < 0.05$, one-way ANOVA analysis of variance followed by Fisher's-multiple range test). (B) The NOX activity was measured at indicated times after cisplatin treatment in LNCaP, PC3 and DU145 cells. Values are mean ± SEM ($n = 9$). Means values with different letters are significantly different ($p < 0.05$, one-way ANOVA analysis of variance followed by Fisher's-multiple range test). (C) The mRNA expression levels of *NOX* isoforms were examined by semi-quantitative RT-PCR. A representative data from three independent experiments is shown.

antioxidants and NOX inhibitors have been shown to induce growth inhibition of cancer cells through scavenging of ROS or inhibition of NOX [2–4]. Kumar et al. [6] reported that antioxidant or NOX inhibitor suppresses cell growth and survival signalling such as extracellular signal-regulated kinase (ERK)1/ERK2, p38 MAP kinase and PI3K/Akt signalling. It was thus assumed that pre-treatment of LNCaP cells with NAC and DPI might have eliminated ROS necessary for cell growth and survival. Landriscina et al. [21] indicated that cancer cells constitute antioxidant networks for maintaining intracellular homeostasis and favouring cell survival. These reports and our data suggest that the balance between intracellular ROS concentration and adaptability to oxidative stress is very important for cell survival.

The NOX family consists of five isoforms (NOX1–5) [22]. In prostate cancer cells, several reports have demonstrated expression profiles of NOX isoforms [6,8,23]. Furthermore, Arbiser et al. [24] described that ectopic expression of NOX1 positively regulates cell growth, tumourigenicity and angiogenicity in prostate cancer cells. Brar et al. [8] demonstrated that NOX5 negatively regulates growth and apoptosis in DU145 cells. We examined the NOX activity as well as mRNA expression of *NOX* isoforms in LNCaP, PC3 and DU145 cells (Figure 5). Although the expression levels of *NOXs* in LNCaP cells were lower than those in PC3 and DU145 cells, the NOX activity in LNCaP cells was higher than PC3 and DU145 cells (Figures 5B and C). NOX is activated through the Rho-like small GTPase Rac1 or Rac2 and PKC [25]. The differences in cisplatin-induced ROS production among cell lines might be due to aberrant upstream signalling rather than distinct expression patterns of NOX isoforms.

Conclusions

We have demonstrated that cisplatin induces production of ROS and H_2O_2 via NOX activation in human prostate cancer cell lines. Cisplatin-induced NOX activation and subsequent ROS and H_2O_2 production were most prominent in hormone-sensitive LNCaP cells compared with hormone-refractory PC3 and DU145 cells, which may in part explain higher chemosensitivity of LNCaP cells to cisplatin.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Jang HJ, Hwang S, Cho KY, Kim do K, Chay KO, Kim JK. Taxol induces oxidative neuronal cell death by enhancing the activity of NADPH oxidase in mouse cortical cultures. Neurosci Lett 2008;443:17–22.

[2] Zi X, Grasso AW, Kung HJ, Agarwal R. A flavonoid antioxidant, silymarin, inhibits activation of erbB1 signaling and induces cyclin-dependent kinase inhibitors, G1 arrest, and anticarcinogenic effects in human prostate carcinoma DU145 cells. Cancer Res 1998;58:1920–1929.

[3] Wang SI, Mukhtar H. Gene expression profile in human prostate LNCaP cancer cells by (-)-epigallocatechin-3-gallate. Cancer Lett 2002;182:43–51.

[4] Wang KD, Tompkins A, Simonyi A, Korthuis RJ, Sun AY, Sun GY. Apocynin protects against global cerebral ischemia-reperfusion-induced oxidative stress and injury in the gerbil hippocampus. Brain Res 2006;1090:182–189.

[5] Dakubo GD, Parr RL, Costello LC, Franklin RB, Thayer RE. Altered metabolism and mitochomdrial genome in prostate cancer. J Clin Pathol 2006;59:10–16.

[6] Kumar B, Koul S, Khandrika L, Meacham RB, Koul HK. Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype. Cancer Res 2008;68:1777–1785.

[7] Vignais PV. The superoxide-generating NAD(P)H oxidase: structural aspects and activation mechanism. Cell Mol Life Sci 2002;59:1428–1459.

[8] Brar SS, Corbin Z, Kennedy TP, Hemendinger R, Thornton L, Bommarius B, et al. NOX5 NAD(P)H oxidase regulates growth and apoptosis in DU145 prostate cancer cells. Am J Physiol Cell Physiol 2003;285:353–369.

[9] Li L, Watts SW, Banes AK, Galligan JJ, Fink GD, Chen AF. NADPH oxidase-derived superoxide augments endothelin-1-induced venoconstriction in mineralocorticoid hypertension. Hypertension 2003;42:316–321.

[10] Wang X, Martindale JL, Liu Y, Holbrook NJ. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signaling pathways on cell survival. Biochem J 1998;333:291–300.

[11] Ripple MO, Henry WF, Rago RP, Wilding G. Effect of antioxidants on androgen-induced AP-1 and NF-κB DNA-binding activity in prostate carcinoma cells. J Natl Cancer Inst 1999;91:1227–1232.

[12] Dolado I, Swat A, Ajenjo N, De Vita G, Cuadrado A, Nebreda AR. p38α MAP kinase as a sensor of reactive oxygen species in tumorigenesis. Cancer Cell 2007;11:191–205.

[13] Zhang H, Mizumachi T, Carcel-Trullols J, Li L, Naito A, Spencer HJ, et al. Targeting human 8-oxoguanine DNA glycosylase (hOGG1) to mitochondria enhances cisplatin cytotoxicity in hepatoma cells. Carcinogenesis 2007;28:1629–1637.

[14] Kirshner JR, He S, Balasubramanyam V, Kepros J, Yang CY, Zhang M, et al. Elesclomol induces cancer cell apoptosis through oxidative stress. Mol Cancer Ther 2008;7:2319–2327.

[15] McLean L, Soto U, Agama K, Francis J, Jimenez R, Pommier Y, et al. Aminoflavone induces oxidative DNA damage and reactive oxidative species-mediated apoptosis in breast cancer cells. Int J Cancer 2008;122:1665–1674.

[16] Shin DY, Shin HJ, Kim GY, Cheong J, Choi IW, Kim SK, et al. Streptochorin isolated from streptomyces sp. Induces apoptosis in human hepatocarcinoma cells through a reactive oxygen species-mediated mitochondrial pathway. J Microbiol Biotechnol 2008;18:1862–1868.

[17] Kruidering M, Van de WB, de Heer E, Mulder GJ, Nagelkerke JF. Cisplatin-induced nephrotoxicity in porcine proximal tubular cells: mitochondrial dysfunction by inhibition of complexes I to IV of the respiratory chain. J Pharmacol Exp Ther 1997;280:638–649.

[18] Zuliani T, Denis V, Noblesse E Schnebert S, Andre P, Dumas M, Ratinaud MH. Hydrogen peroxide-induced cell death in normal human keratinocytes is differentiation dependent. Free Radic Biol Med 2005;38:307–316.

[19] Kojima K, Ohhashi R, Fujita, Hamada N, Akao Y, Nozawa Y, et al. A role for SIRT1 in cell growth and chemoresistance in prostate cancer PC3 and DU145 cells. Biochem Biophys Res Commun 2008;29:423–428.

[20] Iwao-Koizumi K, Matoba R, Ueno N, Kim SJ, Ando A, Miyoshi Y, et al. Prediction of docetaxel response in human breast cancer by gene expression profiling. J Clin Oncol 2005;23:422–431.

[21] Landriscina M, Maddalena F, Laudiero G, Esposito F. Adaptation to oxidative stress, chemoresistance, and cell survival. Antioxid Redox Signal 2009;11:2701–2716.

[22] Bokoch GM, Diebold B, Kim JS, Gianni D. Emerging evidence for the importance of phosphorylation in the regulation of NADPH oxidases. Antioxid Redox Signal 2009;11:2429–2441.

[23] Lim SD, Sun C, Lambeth JD, Marshall F, Amin M, Chung L, et al. Increase NOX1 and hydrogen peroxide in prostate cancer. Prostate 2005;62:200–207.

[24] Arbiser JL, Petros J, Klafter R, Govindajaran B, Mclaghlin ER, Brown LF, et al. Reactive oxgen generated by NOX1 triggers the angiogenic switch. Proc Natl Acad Sci USA 2002;99:715–720.

[25] Hordijk PL. Regulation of NOXs: the role of rac proteins. Circ Res 2006;98:453–462.

This paper was first published online on Early Online on 21 June 2011.

Supplementary material available online

Figure 1 Supplemental Material.