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The Effect of Troglitazone on Thermal Sensitivity in Uterine Cervix Cancer Cells

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Purpose: Troglitazone (TRO), a PPAR-γ agonist, can reduce heat shock protein (HSP) 70 and increase the antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, which might affect thermal sensitivity. Here, we investigated whether TRO modifies thermal sensitivity in uterine cervical cancer cells, which is most commonly treated by hyperthermia (HT).

Materials and Methods: HeLa cells were treated with 5 μM TRO for 24 hours before HT at 42°C for 1 hour. Cell survival was analyzed by clonogenic assay. The expression of HSPs was analyzed by Western blot. SOD and catalase activity was measured and reactive oxygen species (ROS) was measured using 2',7'-dichlorofluorescin diacetate and dihydroethidium.

Results: The decreased cell survival by HT was increased by preincubation with TRO before HT. Expression of HSP 70 was increased by HT however, it was not decreased by preincubation with TRO before HT. The decreased Bcl-2 expression by HT was increased by preincubation with TRO. SOD and catalase activity was increased by 1.2 and 1.3 times,respectively with TRO. Increased ROS by HT was decreased by preincubation with TRO.

Conclusion: TRO decreases thermal sensitivity through increased SOD and catalase activity, as well as scavenging ROS in HeLa cells.

Key Words: Troglitazone, Hyperthermia, Reactive oxygen species

Introduction

Uterine cervix cancer is the second most common cancer among women worldwide, with an estimated 493,000 new cases and 273,000 deaths in 2002.[1] Although the incidence is decreasing in developed countries, cervix cancer remains the most common cancer among women in many developing countries. In almost stages, cervix cancer is commonly treated with radiation therapy.[2] Due to its characteristic cell killing mechanism; more cytotoxic to the cells in synthetic phase and hypoxic cells, hyperthermia (HT) has been much concerned to increase cancer cell killing alone, or combined with radiation therapy (RT) or chemotherapy.[3,4] Among solid tumors, cervix cancer has been studied comprehensively and HT increased local control and survival of locally advanced cervix cancer, when combined with RT.[5~7] Increased oxygenation due to vasodilation by mild hyperthermia (40～42°C) has been suggested as another important mechanism of enhancing the effect of RT through overcoming hypoxic cancer cells.[8)

Heat shock proteins (HSPs) are induced or activated as a molecular chaperone to protect cells from death or protein denaturation, when the cells face to a variety of stresses such as heat, toxic chemicals and oxidative stress.[9,10] HSP expression is regulated through the activity of a transcription factor, heat shock factor 1 (HSF1).[11] Hence, silencing the HSF1 or HSPs may be an effective strategy for the sensitization of

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cancer cells to HT.[12] Because being elevated in many types of cancer and appears to play a role in resistance to HT-induced cell death by apoptosis and other mechanisms, HSP70 has been studied much and regarded as an effective target to reduce thermal resistance.[13,14]

Troglitazone (TRO) is one of the thiazolidinediones which is used to control Type II diabetes.[15] TRO works a part by activating peroxisome proliferator-activated receptor gamma (PPAR-γ), a ligand-activated transcription factor. PPAR-γ is expressed in highest level in adipocytes and involved in the processes of cellular proliferation, differentiation, and apoptosis.[16] PPAR-γ also is expressed in many other tissues and cell types throughout the body, including monocytes and macrophages, liver, skeletal muscle, breast, prostate, colon, and uterine cervix.[16,17] Many studies have shown the PPAR-γ ligands have anticancer activity against a wide variety of cancer cells.[18] Among PPAR-γ ligands TRO has been studied the most comprehensively and demonstrated antiproliferative effect on various types of cancer cells including uterine cervix cancer.[18] From these TRO has been considered as a potential anticancer drug. In addition, TRO decreases HSP 70 protein[19] and induces cellular acidosis.[20,21] The reduction of HSP 70 protein and cellular acidosis by TRO are expected to increase thermal sensitivity.

Reactive oxygen species (ROS) is involved in the cytotoxicity of HT. Thermal stress to cells induces ROS such as superoxide anion ($O2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), nitric oxide ($^{\cdot}NO$), and peroxynitrite ($ONOO^{-}$).[22,23] TRO is known to induce superoxide dismutase (SOD)[24] and catalase.[25] Both SOD and catalase may decrease thermal sensitivity via ROS scavenging. These contradictory effects of TRO on HT have never been reported yet. In addition, most of studies with TRO were performed at relatively high concentrations (20∼50 μM), while the clinically achievable concentrations are around 2∼5 μM.[26] Therefore, we investigated the effect of low dose TRO on HT in cervix cancer cells, which is the most commonly treated with HT.

Materials and Methods

1. Cell culture and chemicals

A human cervical cancer cell line, HeLa was obtained from Korean Cell Line Bank (Seoul, Korea). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO2. FBS, RPMI 1640, trypsin-EDTA and antibiotics for cell culture were purchased from Life Technologies (Carlsbad, CA, USA). TRO was purchased from Cayman Chemical Company (Arbor, MI, USA) and dissolved in dimethylsulfoxide as a vehicle. 2’ 7’-dichlorofluorescein diacetate (H_2DCFH-DA) was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Annexin Ⅴ fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Becton Dickinson (San Jose, CA, USA). Antibodies against HSP 27, Bcl-2, Bax as well as secondary AP conjugated antibodies were purchased from Santa Curz Biotechnology (Santa Curz, CA, USA) and antibodies against HSP 70 and HSP 90 were purchased from StressGen Biotechnologies (Victoria, BC, Canada).

2. Clonogenic assay

HeLa cells were seeded on cell culture dishes and incubated overnight for the cells to attach on the dishes. After incubated with TRO for 24 hours the cells were treated with heat (42°C for 1 hour). The cells were trypsinized to generate single cell suspension and seeded into 60 mm dishes with 500 to 700 cells per dish in triplicate. The cells were incubated for 7∼14 days at 37°C in 5% CO2. Colonies were stained with 0.5% crystal violet and absolute ethanol, at least 50 or more cells were counted as a colony. Surviving fraction (SF) was calculated as (mean colonies count)/(cells plated)×(plating efficiency), where plating efficiency was defined as (mean colonies counted)/(cells plated) for control.

3. Western blot

The level of expression of proteins was determined in control and treated cells by Western blotting. Cells were treated with TRO for 24 hours and HT of 42°C for 1 hour. Then cells were washed twice in phosphate buffered saline (PBS) and harvested by lysis buffer and sonicated in buffer containing protease inhibitors. Total protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA, USA). Thirty μg of protein was separated on 10∼12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidine difluoride (PVDF) membranes (Bio-Rad). The membranes were blocked with 5% non-fat dried milk at room temperature for 45 min and

incubated with primary antibody overnight with constant agitation at 4°C. Anti-HSP 70, HSP 90 and HSP 27 were diluted 1 : 3,000 in Tris-Buffered Saline Tween-20 (TBS-T). After washed three times with TBS-T the membranes were incubated with second antibody for 1 hour at room temperature with constant agitation. The detections were visualized using an enhanced chemiluminescence reagent (Intron biotechnology, Seongnam, Korea).

4. Measurement of SOD and catalase activity

SOD was measured using a kit (Dojindo Molecular Technologies, Rockville, MD, USA) according to a manufacturer's instruction. Catalase activity was quantitated spetrophotometrically following the decomposition of H_2O_2 at 240 nm. The catalase activity was expressed as U/mg protein.

5. Measurement of ROS

First, to measure ROS a fluorescent probe, H_2DCFH-DA was used. DCFH-DA is known to be sensitive to a broad range of intracellular oxidative stress including $O2^{\cdot -}$, H_2O_2, $^{\cdot}$OH, $^{\cdot}$NO and ONOO[2-3]. H_2DCFH-DA is changed to DCFH by the sodium hydroxide and DCFH is oxidized to 2'7'-DCF by ROS. To measure specifically the level of cellular $O2^{\cdot -}$, the dye DHE was used. $O2^{\cdot -}$ oxidizes DHE to red-fluorescent ethidium (Eth), which can be detected by flow cytometry at 488/620 nm. After incubated with TRO for 24 hours the cells were treated with HT at 42°C for 1 hour. The cells were washed with PBS twice and were incubated with 10 μM of DCFH-DA or 5 μM of DHE in PBS at 37°C for 15 minutes. Again the cells were washed with PBS twice, suspended by trypsinization. Then 2 μl of PI was added for living cell measurement. The fluorescence intensity was measured by a fluorescence microscope (Olympus, Tokyo, Japan) and a flowcytometer (FACS Calibur-S, Becton-Dickinson, San Jose, CA, USA).

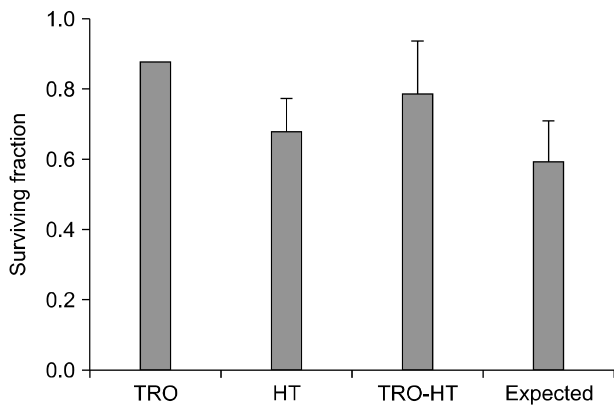

Fig. 1. Effect of troglitazone (TRO; 5 μM for 24 hours) and hyperthermia (HT; 42°C for 1 hour) on the clonogenic survival of HeLa cells. Data indicate surviving fraction as means±SD of three independent experiments. The expected value was calculated from the simple multiplication of surviving fractions of TRO and HT alone.

Results

1. Clonogenic cell survival

First, to know the combined effect of TRO and HT on cell death, clonogenic assay was done. The SFs of 5 μM TRO for 24 hours alone and HT alone (42°C, 1 hour) were 0.88 and 0.68 respectively. When the cells were preincubated with TRO for 24 hour and treated with HT the SF was 0.78. This is higher than that of expected value (0.59), which is calculated by simply multiplying SFs of TRO and HT alone (Fig. 1). This indicates the protective effect of TRO on the cytotoxicity of HT.

2. Effect of TRO on the HSPs and apoptotic proteins

To know the thermoprotective mechanism of TRO we observed the expression change of HSPs in cells treated without or with TRO before HT by Western blot. First, we observed the expression pattern of HSPs according to time elapse after HT alone. There was no change in the expression of HSP 27 and 90 after HT. In the other hand, HSP 70 was increased at

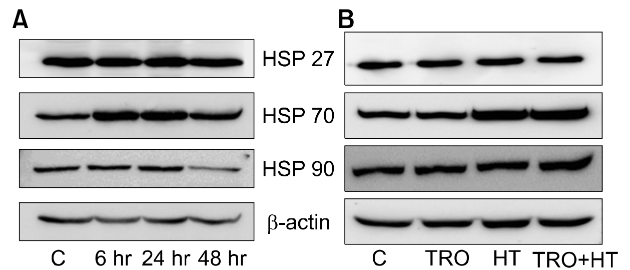

Fig. 2. (A) The expression of heat shock proteins (HSPs) according to time elapse after hyperthermia (HT) 42°C 1 hour in HeLa cells. (B) The expression of HSPs 6 hours after HT 42°C 1 hour in HeLa cells preincubated with or without troglitazone (TRO). These are representatives of three independent experiments.

6 hours and 24 hours after HT (Fig. 2A). However, the increased HSP 70 by HT was not decreased by the preincubation of cells with TRO (Fig. 2B) and also HSP 70 was not changed with TRO alone. This indicates that low dose TRO does not have an effect on HSP 70 expression in HeLa cells. Next, we observed the expression change of apoptosis-related proteins, Bcl-2 and Bax. An antiapoptotic protein, Bcl-2 was decreased by HT and this was increased by preincubation with TRO. On the contrary, a proapoptotic protein, Bax was increased by HT and this was decreased by preincubation with TRO (Fig. 3). This indicates that TRO decreases the cytotoxicity of HT through modulation of apoptotic proteins, that is, increased antiapoptotic protein (Bcl-2) and decreased proapoptotic protein (Bax).

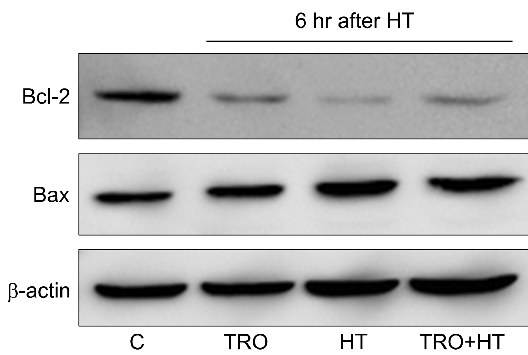

Fig. 3. Expression of apoptotic proteins. The expression of Bcl-2 and Bax 6 hours after hyperthermia (HT) 42°C 1 hour in HeLa cells preincubated with or without troglitazone (TRO). These are representatives of three independent experiments.

3. Effect of TRO on the activity of SOD and catalase

Next, we measured the antioxidant enzymes; SOD and catalase, which may be involved in the removal of ROS produced by HT. The SOD and catalase activity was increased 1.2 times and 1.3 times, respectively with 5 μM of TRO for 24 hours (Fig. 4).

4. Effect of TRO on the removal of ROS increased by HT

The mean fluorescent intensities (MFIs) were measured using DCFH-DA and DHE. Both of the MFI of DCF (Fig. 5) and Eth (Fig. 6) was increased significantly by HT. To know whether TRO contributes to scavenge ROS, cells were preincubated with TRO before HT and the MFIs were measured. When the cells were preincubated with TRO before HT both of the MFIs of DCF and Eth were decreased compared with HT alone (Figs. 5 and 6). The change of fluorescence intensity was confirmed with a fluorescence microscope (Fig. 7). SOD dismutase $O2^{\cdot}$ to H_2O_2, which is converted into O2 and water by catalase. To verify that increased catalase by TRO contributes to remove ROS increased by HT, the cells were incubated with TRO and 0.1 mM of 3-amino-1, 2, 4-triazole (ATZ), a chemical inhibitor of catalase and ROS was measured. The decreased ROS by TRO was increased by the addition of ATZ (Figs. 5 and 6). This means that increased

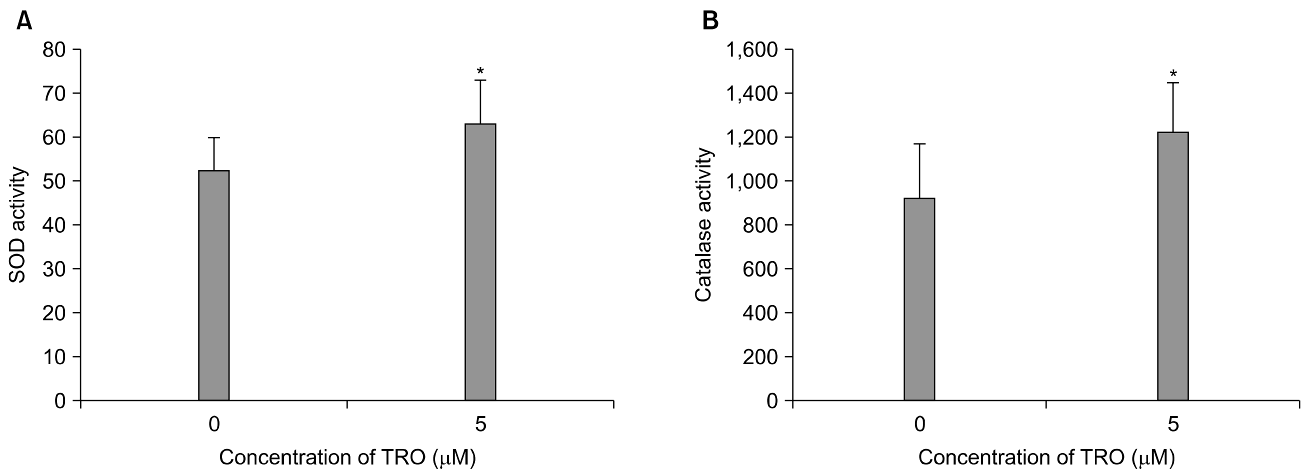

Fig. 4. Superoxide dismutase (SOD) (A) and catalase (B) activities after treated with 5 μM troglitazone (TRO) for 24 hours. Activity is expressed as unit per mg of protein (u/mg), and means±SD. *$p<0.05$, compared with control.

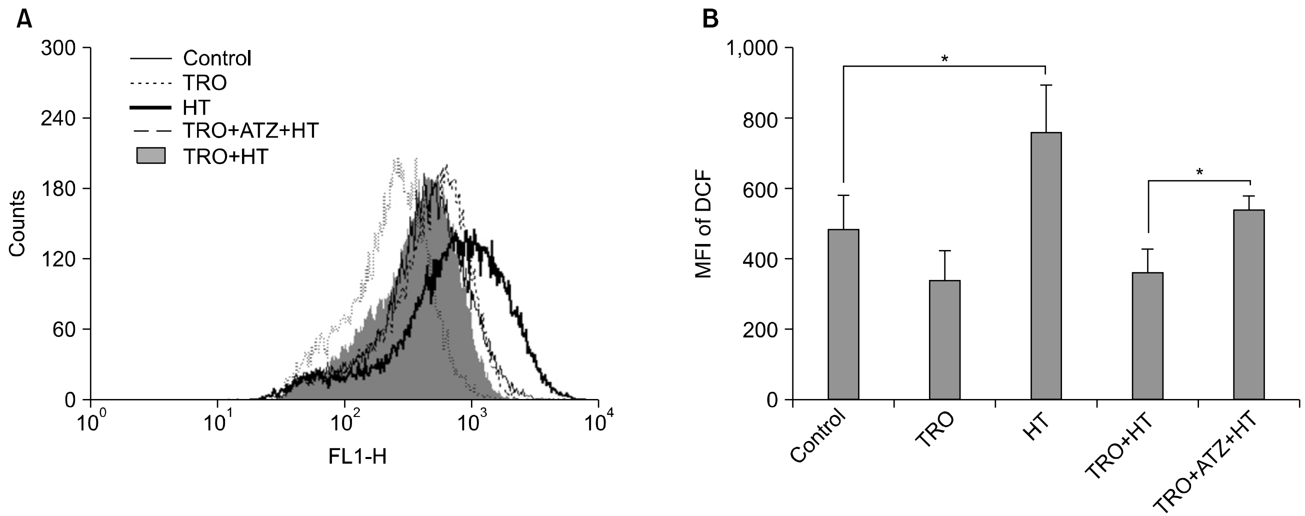

Fig. 5. The change of reactive oxygen species (ROS) measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) after hyperthermia (HT) according to preincubation with troglitazone (TRO) and 3-amino-1, 2, 4-triazole (ATZ) or not in HeLa cells. (A) A representative histogram of three independent experiments. (B) The mean fluorescent intensity (MFI) is expressed as artificial unit and means±SD. *$p < 0.05$.

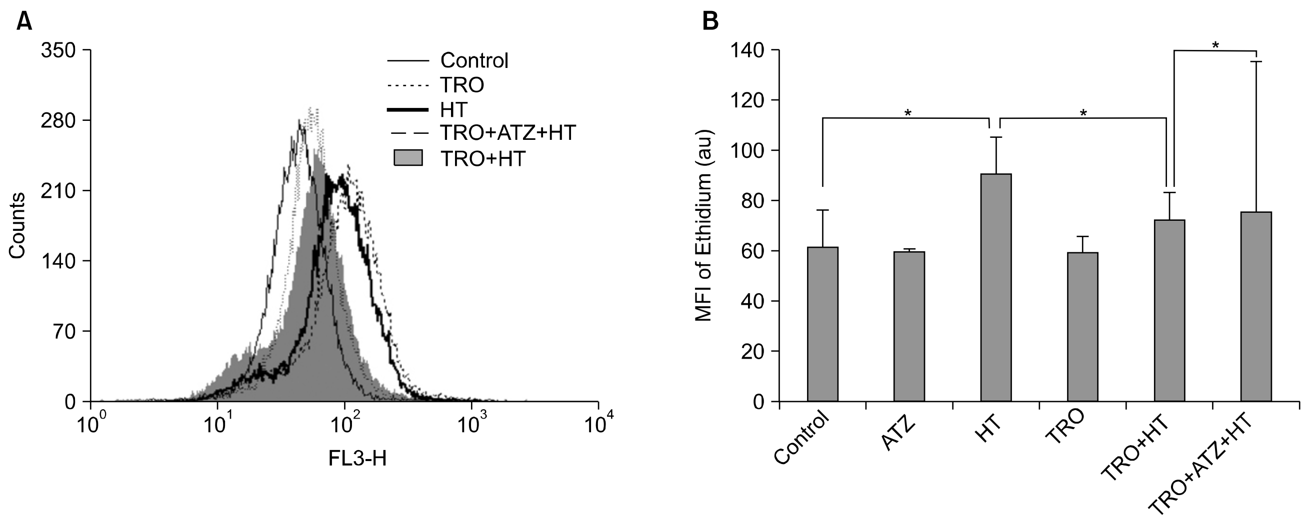

Fig. 6. The change of reactive oxygen species (ROS) measured using DHE after hyperthermia (HT) according to preincubation with troglitazone (TRO) and 3-amino-1, 2, 4-triazole (ATZ) or not in HeLa cells. (A) A representative histogram of three independent experiments. (B) The mean fluorescent intensity (MFI) is expressed as artificial unit and means±SD. *$p < 0.05$.

catalase by TRO contributes to remove the ROS increased by HT.

Discussion and Conclusion

In this study we investigated the effect of clinically applicable dose TRO on HT in cervix cancer cells. The results showed TRO decreases thermal sensitivity by scavenging ROS through increased SOD and catalase activity. The increase of intracellular ROS by HT is considered to be a major mechanism for HT-induced cellular responses, including signal transduction, ubiquitin pathway, cytoskeletal changes, cell cycle regulation and apoptosis.[3,27] There has been a controversy as to how and whether heat stress induces H_2O_2 or $O_2^{\cdot}$.[22] In this study the MFI of DCF, an indicator of a broad range of intracellular oxidative stress and the MFI of Eth, an indicator of $O2^{\cdot}$ was increased by HT. Complexes I and III of the mitochondrial electron transport chain (ETC) are the

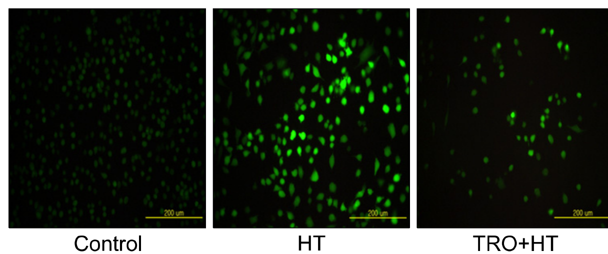

Fig. 7. The change of reactive oxygen species labeled with 2′7′-dichlorofluorescein diacetate (H_2DCFH-DA) and taken using a fluorescence microscope in HeLa cells after hyperthermia (HT) preincubated with troglitazone (TRO) or not. These are representatives of three independent experiments.

two major sites of ROS production under physiological conditions.[28,29] Heat stress increases the electron (e^-) leak from the ETC and thus the level of $O_2^{\cdot}$ (a reaction product of e^- and O_2) will be increased.[23] Endogenous or exogenous ROS will induce apoptosis and necrosis. Thermal and oxidative stresses upregulate the inducible HSPs, especially HSP70 and HSP27, which protect cells from deleterious effects through their chaperoning activity[30] and suppress the function of mitochondrial permeability transition pore opening.[31] At the postmitochondrial level, HSP70 binds to Apaf-1, thereby preventing the recruitment of procaspase-9 to apoptosome,[32] and HSP27 binds to released Cytc, thus blocking the Apaf-l/procaspase-9 interaction.[33] Therefore, those HSPs attenuate mitochondria-mediated apoptosis. In this study HSP70 was increased, but HSP 27 and 90 was not increased by HT. From a previous report[26] we expected TRO will reduce the increased HSP70 by HT. On the contrary to our expectation, preincubation of TRO did not affect on the expression of HSP70 by HT. This discrepancy is thought to be related with the differences of cell types and the concentration of TRO. In this study we used lower dose (5 μM) TRO compared with 50 μM of another.[26] TRO has been reported to have pro-oxidant[34] or anti-oxidant activity.[35,36] This contradictory activity of TRO seems to be dependent on cell types and the concentration of TRO. Usually in higher concentration TRO has a cytotoxic effect, which is accompanying increased ROS.

In this study we used low dose of TRO to know whether TRO can be used to modify a clinical effect of HT. The antioxidant activity of low dose TRO reduced the cytotoxic effect of HT in cervix cancer cells.

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국문초록

자궁 경부암 세포에서 Troglitazone이 온열감수성에 미치는 영향

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목 적: Troglitazone (TRO)은 PPAR-γ 작동제로서 온열감수성의 결정에 중요한 요인인 heat shock protein (HSP) 70의 합성을 저해하고 superoxide dismutase (SOD)와 카타라제를 증가시키는 것으로 알려져 있다. 이에 자궁경부암 세포를 대상으로 TRO가 온열감수성에 미치는 영향을 연구하였다.

대상 및 방법: HeLa 세포를 5 μM TRO로 24시간 처치한 후 42°C에서 1시간 동안 온열처리를 시행하였다. 세포 생존 분획은 clonogenic assay로 측정하였다. 단백질 발현의 변화는 Western blot으로 분석하였다. SOD와 카타라제의 활성도를 측정하였으며, reactive oxygen species (ROS) 는 2',7'-dichlorofluorescin diacetate와 dihydroethidium를 사용하여 측정하였다.

결 과: 온열처리에 의해 감소된 생존분획이 TRO 전처치에 의해 증가하였다. 온열처리에의해 HSP 70의 발현은 증가하였으나 TRO 전 처치에 의해 감소되지는 않았다. SOD와 카타라제의 활성도가 각각 1.2배, 1.3배 증가하였다. 온열처리에 의해 ROS가 증가하였으며, 증가된 ROS는 TRO 전 처치에 의해 감소하였다.

결 론: TRO는 SOD와 카타라제의 활성도를 증가시키며 이는 온열에 의한 ROS를 감소시켜 결과적으로 온열감수성을 저하시킨다.

핵심용어: 트로글리타존, 온열치료, 반응성산소종