Nitration of PPARγ inhibits ligand-dependent translocation into the nucleus in a macrophage-like cell line, RAW 264

Atsuhito Shibuya[a,b], Koichiro Wada[a,*], Atsushi Nakajima[c], Makio Saeki[a], Kazufumi Katayama[d], Tadanori Mayumi[d], Takashi Kadowaki[e], Hitoshi Niwa[b], Yoshinori Kamisaki[a]

[a] *Department of Pharmacology, Graduate School of Dentistry, Osaka University, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan*
[b] *Department of Dental Anesthesiology, Graduate School of Dentistry, Osaka University, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan*
[c] *The Third Department of Internal Medicine, Yokohama City University School of Medicine, 3-9 Fuku-ura, Yokohama 236-0004, Japan*
[d] *Department of Biopharmaceutics, Graduate School of Pharmaceutics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan*
[e] *Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*

Received 22 June 2002; accepted 24 June 2002

First published online 11 July 2002

Edited by Barry Halliwell

Abstract **Nitration of tyrosine residues in proteins has been observed in many inflammatory tissues of arthritis, ulcerative colitis, septic shock and ischemia-reperfusion injury. Although several studies have been carried out, it is still unclear what type of protein is nitrated and whether tyrosine nitration interferes with protein function. Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor whose activation is linked to several physiological pathways including regulation of insulin sensitivity and control of inflammation. PPARγ possesses several tyrosine residues, which might be potential targets for nitration by peroxynitrite during inflammatory responses. Here we have investigated whether PPARγ is nitrated in macrophage-like RAW 264 cells and the effect of nitration on the translocation of PPARγ into the nucleus. Western blot analysis showed that tumor necrosis factor-α, lipopolysaccharide or peroxynitrite treatment significantly increases the nitration of PPARγ. Cell fractionation analysis and immunofluorescence coupled with confocal laser microscopy revealed that nitration of PPARγ inhibits its ligand-dependent translocation from the cytosol into the nucleus. Together, these results indicate that nitration of PPARγ during inflammation may be involved in a reduction in the control of inflammatory responses and also in the development of resistance to PPARγ ligand-based therapies against inflammation.**

Key words: PPARγ; Nitrotyrosine; Macrophage; Translocation; Inflammation

1. Introduction

Nitration of tyrosine residues in proteins is observed in many diseases such as arthritis, nephritis, ulcerative colitis, ischemia-reperfusion injury, septic shock, and neuronal disorders [1–6]. Most reports have indicated that increased formation of nitrotyrosine is a marker of inflammation in tissues, because nitrotyrosine is formed through the reaction of a tyrosine residue with peroxynitrite that is generated from superoxide and nitric oxide (NO) radicals [7,8]. Although the pathological or physiological roles of nitrotyrosine are still unknown, several studies have reported that nitrotyrosine formation may alter the function of proteins in signal transduction pathways [9–12].

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the nuclear receptor superfamily of ligand-activated transcriptional factors and plays a key role in adipocyte differentiation and insulin sensitivity [13–15]. Synthetic ligands of PPARγ, such as troglitazone, pioglitazone and rosiglitazone (Rosi), are used as oral anti-hyperglycemic agents in therapy of non-insulin-dependent diabetes mellitus. Recent studies have shown that, in addition to its classical role, PPARγ may also be involved in the control of inflammation, in particular in modulating the production of inflammatory mediators [16,17]. We previously reported that the endogenous PPARγ pathway acts against inflammation in the gastrointestinal tract in animal models of inflammatory bowel disease, such as dextran sodium sulfate- and ischemia-induced colitis [18–20]. Therefore, the endogenous PPARγ pathway is considered a preventive system for excessive inflammation, and thus dysfunction or suppression of PPARγ may cause aggravation of chronic or recurrent inflammation.

Here we have focused on the nitration of PPARγ proteins in the macrophage-like cell line RAW 264 and investigated whether PPARγ functions are affected by nitration. Macrophage cells are considered key players in inflammation and highly express PPARγ proteins, therefore, it is important to investigate the effect of alterations in the function of the PPARγ pathway in these cells. In this study, nitration of PPARγ in RAW 264 cells was induced by stimulation with peroxynitrite, lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α). We report that the stimulation of RAW 264 cells caused strong nitration of PPARγ, which in turn inhibited its ligand-dependent translocation from the cytosol into the nucleus.

2. Materials and methods

2.1. Reagents and antibodies

The PPARγ-specific ligand, Rosi, was a kind gift from Glaxo SmithKline (Tokyo, Japan). Anti-PPARγ polyclonal and monoclonal antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-nitrotyrosine monoclonal antibody was purchased from Zymed (Zymed Laboratories Inc., CA, USA). LPS and TNF-α were pur-

*Corresponding author. Fax: (81)-6-6879 2914.
E-mail address: kwada@dent.osaka-u.ac.jp (K. Wada).

0014-5793/02/$22.00
PII: S0014-5793(02)03059-4

chased from Sigma (Tokyo, Japan). Peroxynitrite solution was purchased from Dojindo (Kumamoto, Japan). All other chemicals were reagent grade.

2.2. Cell culture and ligand treatment

RAW 264 cells, a murine peritoneal macrophage-like cell line, were purchased from RIKEN GenBank (Tokyo, Japan). The cells were cultured to confluence in Dulbecco's modified Eagle's medium (Sigma, Tokyo, Japan) supplemented with penicillin (5 U/ml), streptomycin (5 μg/ml) and amphotericin B (250 ng/ml) in 10 cm dishes at 37°C under 5% CO_2. RAW 264 cells were stimulated by TNF-α (50 ng/ml), LPS (10 μg/ml) or vehicle for 18 h, incubated with or without PPARγ ligand (Rosi, 30 μM) for 3 h, and then harvested. For peroxynitrite, stimulation was performed for 40 min, followed by incubation with PPARγ ligand as described above.

2.3. Preparation of cell extracts and fractionation

Cells were homogenized in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 4 mM EGTA, 1% Triton X-100) containing a cocktail of protease inhibitors (Sigma, Tokyo, Japan). Cell extracts were prepared by a centrifugation at 16 000×*g*, and used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Immunoprecipitation was carried out overnight by rotation with anti-PPARγ polyclonal antibody crosslinked to Protein G-Sepharose beads (Amersham Pharmacia Biotech Japan, Tokyo, Japan) [21]. Cytosol and nuclear fractions were prepared using NE-PER Nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

2.4. Western blot analysis

Samples (each containing 20 μg protein) were resolved by SDS–PAGE and transferred onto a polyvinylidene difluoride membrane. Anti-nitrotyrosine antibody, anti-PPARγ monoclonal or polyclonal antibody were applied overnight at 4°C. Immunoreactivity was detected with an ECL-plus kit (Amersham, London, UK).

2.5. Confocal microscopy

RAW 264 cells were cultured on a Lab-Tek II chamber slide (Nalge Nunc International, Naperville, IL, USA). Four variations of stimulation and ligand treatment were performed as follows: no stimulation plus vehicle (no ligand), no stimulation plus ligand (Rosi, 30 μM), LPS stimulation plus Rosi and TNF-α stimulation plus Rosi. Stimulation with TNF-α or LPS was carried out for 18 h, followed by treatment with Rosi (30 μM) for 3 h. The cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized in PBS containing 0.1% Triton X-100 for 30 min and rinsed with PBS. The fixed cells were incubated with anti-PPARγ rabbit polyclonal antibody (1:1000) for 16 h at 4°C and then with Alexa Fluor 488 goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA) for 1 h. Nuclear staining was performed by incubation with SYTOX Orange (Molecular Probes, Eugene, OR, USA). A confocal laser-scanning microscope (MRC1024, Bio-Rad, Tokyo, Japan) was used to visualize the localization of PPARγ.

3. Results

3.1. PPARγ proteins are strongly nitrated by peroxynitrite or LPS

We previously observed that peroxynitrite treatment (500 μM, 40 min) in RAW 264 cells induced the nitration of tyrosine residues in several proteins, including proteins with molecular weights of <37, 50, 70, 100–110 and 150–180 kDa [22]. Although these proteins have not been identified, we focused on the 50-kDa protein, which was strongly nitrated by peroxynitrite (Fig. 1, upper panel). In particular, the molecular weight of PPARγ is 50 kDa and we therefore investigated whether PPARγ is the 50-kDa nitrated protein.

We immunoprecipitated the nitrated proteins using anti-PPARγ polyclonal antibody and analyzed them by Western blot using anti-nitrotyrosine monoclonal antibody. Fig. 2 shows the nitration of immunoprecipitated and non-immunoprecipitated proteins from peroxynitrite-treated or LPS-stimulated RAW 264 cells. Although peroxynitrite or LPS caused the nitration of several proteins in non-immunoprecipitated samples (no IP in Fig. 2A), the proteins immunoprecipitated by anti-PPARγ antibody were strongly nitrated (IP in Fig. 2A). We examined a competitive blocking of nitrated signals to anti-nitrotyrosine antibody by an excessive amount of 3-nitrotyrosine to verify the immunostaining of nitrated proteins. As shown in Fig. 2B, the nitrated signals to anti-nitrotyrosine antibody were diminished by the presence of 3-nitrotyrosine. These results indicate that those immunoreactive proteins to anti-nitrotyrosine antibody are nitrated proteins.

We also confirmed that the protein amount of PPARγ was not altered by the treatment (Fig. 1, lower panel). These results clearly indicate that PPARγ proteins are strongly nitrated after stimulation with LPS or peroxynitrite in RAW 264 cells.

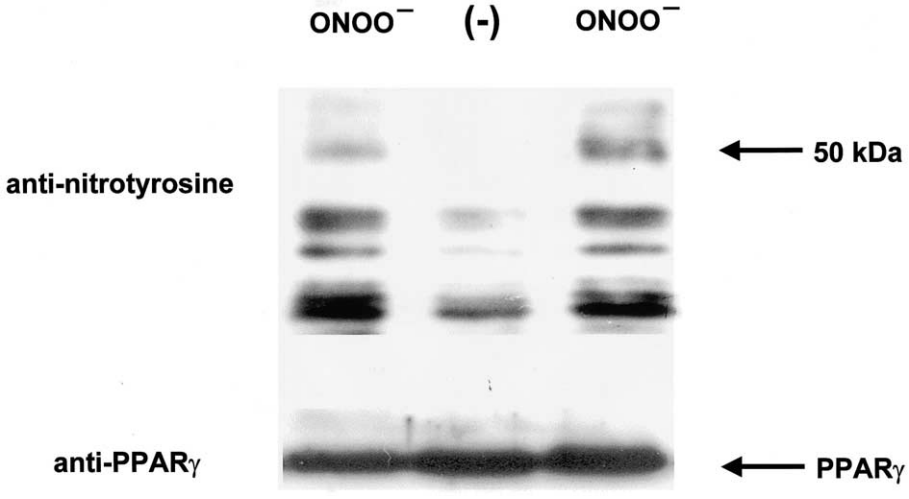

Fig. 1. Nitration of PPARγ in RAW 264 cells by peroxynitrite. Western blot analysis using anti-nitrotyrosine (upper panel) or anti-PPARγ (lower panel) antibody was applied to cell extracts from RAW 264 cells treated with peroxynitrite ($ONOO^-$, 500 μM, 40 min). Although several proteins were nitrated, 50-kDa PPARγ protein was strongly nitrated (arrow in upper panel). Treatment with peroxynitrite did not alter the protein amounts of PPARγ protein (lower panel).

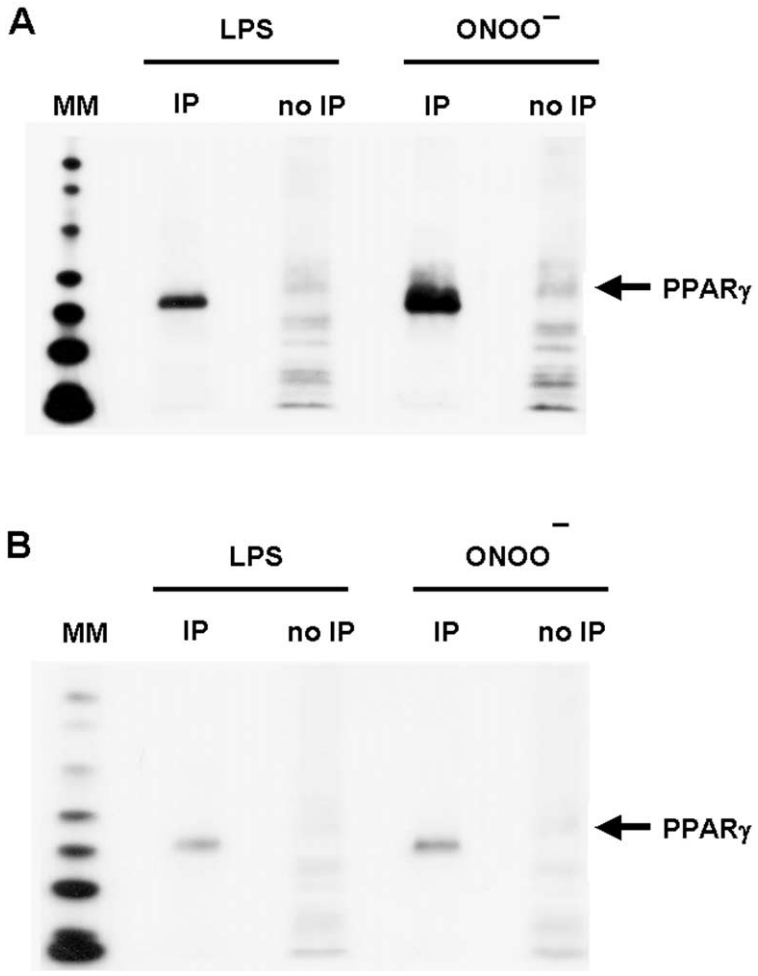

Fig. 2. Nitration of proteins immunoprecipitated by anti-PPARγ antibody from peroxynitrite ($ONOO^-$)- or LPS-stimulated RAW 264 cells. A: Immunoprecipitated (IP) or non-immunoprecipitated (no IP) samples were applied to SDS–PAGE and then analyzed for immunoreactivity to anti-nitrotyrosine antibody. Arrows indicate PPARγ. B: Competitive blocking of nitrotyrosine signals by 3-nitrotyrosine. Immunoreaction to anti-nitrotyrosine antibody was performed with the presence of 3-nitrotyrosine (10 mM). MM indicates molecular markers.

3.2. *Nitration of PPARγ inhibits ligand-induced translocation into the nucleus*

To investigate whether nitration of PPARγ may alter the function as a receptor and transcriptional regulator, we examined the ligand-dependent translocation of PPARγ into the nucleus in RAW 264 cells under the normal and nitrated conditions. RAW 264 cells were stimulated with or without LPS or TNF-α, and then incubated with PPARγ ligand (Rosi) or vehicle. Subsequently, cytosol and nuclear fractions were prepared and applied to Western blot analysis using anti-PPARγ antibody. In non-stimulated RAW 264 cells, PPARγ was mainly observed in the cytosol fraction. After treatment with the ligand (Rosi, 30 μM), however, PPARγ was observed in the nuclear fraction (Fig. 3). By contrast, in RAW 264 cells

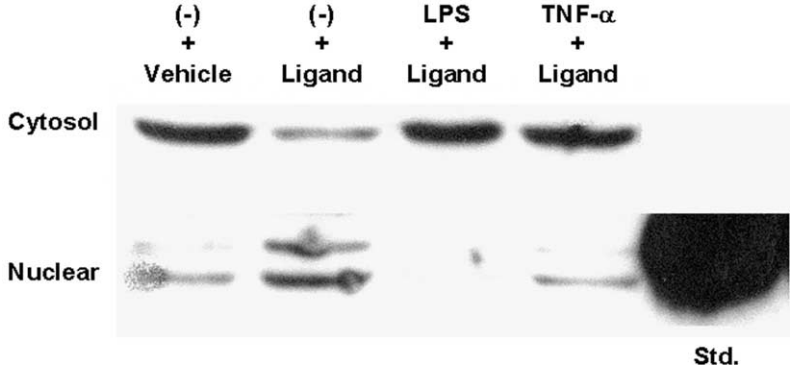

Fig. 3. Ligand-dependent translocation of PPARγ from the cytosol into the nucleus. RAW 264 cells were treated with (LPS or TNF-α) or without (−) stimulant for 18 h, and then incubated with the ligand (Rosi, 30 μM) for 3 h. Cytosol and nuclear fractions were analyzed by anti-PPARγ antibody. Std indicates control PPARγ protein.

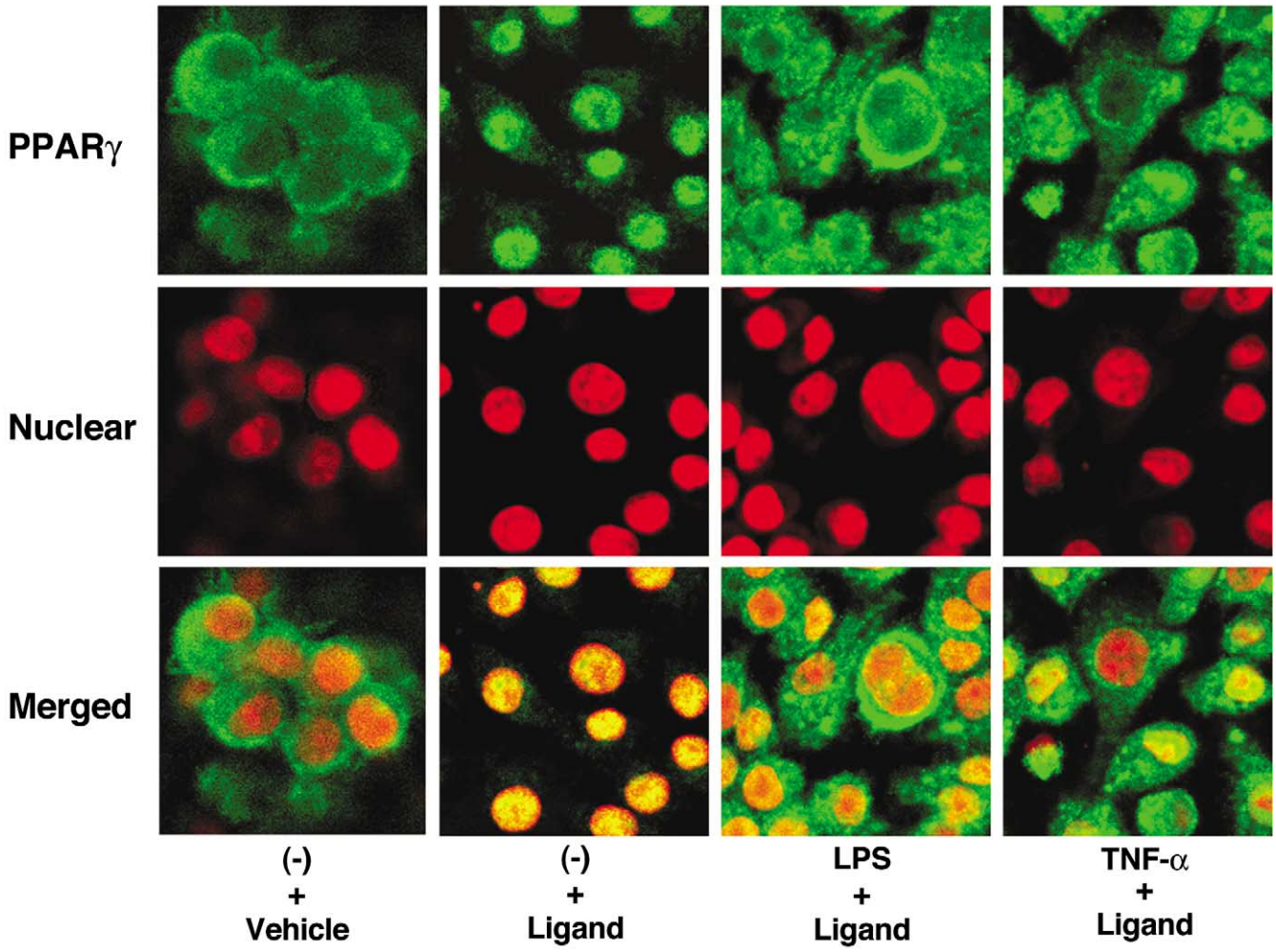

Fig. 4. Visualization of ligand-dependent translocation of PPARγ by confocal laser microscopy. RAW 264 cells were treated with (LPS or TNF-α) or without (−) stimulant and PPARγ ligand as described in the legend of Fig. 3. The fixed cells were treated with anti-PPARγ polyclonal antibody, followed by Alexa Fluor 488 goat anti-rabbit IgG antibody. Nuclear staining was performed by SYTOX Orange. Green color represents PPARγ (upper panels); red color represents nucleus (middle panels). Merged pictures of green and red colors are shown in the lower panels.

stimulated with LPS or TNF-α, nitrated PPARγ remained in the cytosol after treatment with PPARγ ligand (Fig. 3).

To confirm these results, we examined the ligand-dependent translocation of PPARγ into the nucleus using confocal laser microscopy. As shown in Fig. 4, the localization of PPARγ in RAW 264 cells was visualized by immunofluorescence using anti-PPARγ polyclonal antibody. Although in non-stimulated RAW 264 cells PPARγ was translocated from the cytosol into

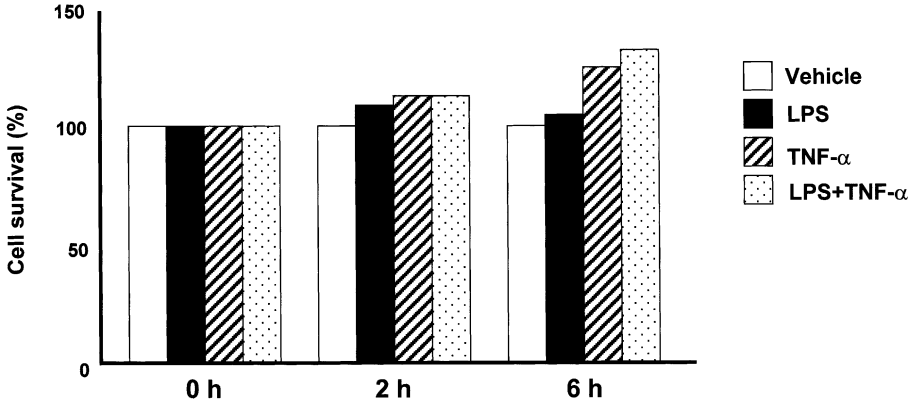

Fig. 5. Cell survival rate under the experimental conditions. Cells were stimulated by LPS, TNF-α, LPS plus TNF-α or vehicle for 0–6 h, and trypan blue dye excretion was measured. The percentage of surviving cells compared with that of vehicle-treated control cells is shown from three independent experiments.

the nucleus by treatment with Rosi, this ligand-dependent translocation was inhibited in RAW 264 cells stimulated with LPS or TNF-α. These results indicate that nitration of PPARγ may inhibit its function as a receptor and transcriptional regulator.

To rule out the possibility that loss of PPARγ translocation is caused by a loss of cell viability, we measured cell survival and proliferation rates in our experimental conditions by trypan blue excretion and MTT assay. As shown in Fig. 5, trypan blue excretion showed that there is no significant difference between control cells and treated cells, although cells treated with LPS plus TNF-α showed a slightly increased cell survival rate. Similar results were obtained with the MTT assay (data not shown). These results clearly rule out the possibility that loss of PPARγ translocation was caused by loss of cell viability.

4. Discussion

It is well known that superoxide and NO radicals react rapidly to form peroxynitrite, which then attacks tyrosine residues in proteins to form nitrotyrosine [1,7]. Although many studies have reported that increased formation of nitrotyrosine is a biomarker for inflammation [2–5], few studies have identified functional alterations in the nitrated proteins. Recently, nitration has been reported to cause alterations in the tyrosine kinase pathway [10–12]. However, no studies have examined the effects of the nitration of nuclear receptors on their functions in inflammatory cells.

In the present study, we observed the selective nitration of PPARγ in stimulated macrophage-like RAW 264 cells. Although several proteins in RAW 264 cells were nitrated by LPS, TNF-α or peroxynitrite, PPARγ was strongly nitrated. This is the first report to identify the specific nitration of PPARγ. PPARγ proteins have many tyrosine residues [23], which may be potential targets for nitration by peroxynitrite or other radicals during inflammatory responses.

In addition, we investigated the functional effect of the nitration of PPARγ in RAW 264 cells. PPARγ is the member of the nuclear receptor superfamily and plays a key role in adipocyte differentiation, insulin sensitivity and anti-inflammation [13–20]. Activation of the PPARγ pathway starts in the cytosol by the binding of specific ligands to PPARγ, which subsequently translocates into the nucleus; we therefore thought ligand-dependent translocation from the cytosol into the nucleus might be affected by tyrosine nitration. Indeed, as clearly shown in Figs. 3 and 4, ligand-dependent translocation was not observed when PPARγ had been previously nitrated by LPS, TNF-α or peroxynitrite. Moreover, we showed that the loss of PPARγ translocation was not due to cell death or loss of viability.

Because the endogenous PPARγ pathway is considered a preventive system to avoid excessive inflammatory responses, the dysfunction or suppression of the PPARγ pathway may cause aggravation of chronic or recurrent inflammation. Our results therefore indicate that nitration of PPARγ during inflammation may participate in sustained inflammation and may also explain the resistance to PPARγ ligand-based therapies against inflammation. Further investigations are required to clarify mechanistic details.

In conclusion, PPARγ was selectively nitrated following inflammatory stimulation in RAW 264 cells, which subsequently inhibited its ligand-dependent translocation into the nucleus. These results indicate that nitration of PPARγ during inflammation may result in increased inflammation, owing to a reduction in the anti-inflammatory effect of the PPARγ pathway.

References

[1] Halliwell, B. (1997) FEBS Lett. 411, 157–160.
[2] Wada, K., Kamisaki, Y., Ohkura, T., Kanda, G., Nakamoto, K., Kishimoto, Y., Ashida, K. and Itoh, T. (1998) Am. J. Physiol. 274, G465–G471.
[3] Bian, K. and Murad, F. (2001) Free Radic. Biol. Med. 31, 421–429.
[4] Mapp, P.I., Klocke, R., Walsh, D.A., Chana, J.K., Stevens, C.R., Gallagher, P.J. and Blake, D.R. (2001) Arthritis Rheum. 44, 1534–1539.
[5] Noiri, E., Nakao, A., Uchida, K., Tsukahara, H., Ohno, M., Fujita, T., Brodsky, S. and Goligorsky, M.S. (2001) Am. J. Physiol. 281, F948–F957.
[6] Pfeiffer, S., Lass, A., Schmidt, K. and Mayer, B. (2001) FASEB J. 15, 2355–2364.
[7] Xia, Y. and Zweier, J.L. (1997) Proc. Natl. Acad. Sci. USA 94, 6954–6958.
[8] Aulak, K.S., Miyagi, M., Yan, L., West, K.A., Massillon, D., Crabb, J.W. and Stuehr, D.J. (2001) Proc. Natl. Acad. Sci. USA 98, 12056–12061.
[9] Kamisaki, Y., Wada, K., Bian, K., Balabanli, B., Davis, K., Martin, E., Behbod, F., Lee, Y.C. and Murad, F. (1998) Proc. Natl. Acad. Sci. USA 95, 11584–11589.
[10] Greenacre, S.A. and Ischiropoulos, H. (2001) Free Radic. Res. 34, 541–581.
[11] Llovera, M., Pearson, J.D., Moreno, C. and Riveros-Moreno, V. (2001) Br. J. Pharmacol. 132, 419–426.
[12] Mallozzi, C., Stasi, A.M. and Minetti, M. (2001) FEBS Lett. 503, 189–195.
[13] Michalik, L. and Wahli, W. (1999) Curr. Opin. Biotechnol. 6, 590–595.
[14] Vamecq, J. and Latruffe, N. (1999) Lancet 354, 141–148.
[15] Kadowaki, T. (2000) J. Clin. Invest. 106, 1305–1307.
[16] Jiang, C., Ting, A. and Seed, B. (1998) Nature 391, 82–86.
[17] Ricote, M., Li, A.C., Willson, T.M., Kelly, J. and Glass, C.K. (1998) Nature 391, 79–82.
[18] Nakajima, A., Wada, K., Miki, H., Kubota, N., Nakajima, N., Terauchi, Y., Ohnishi, S., Saubermann, L.J., Kadowaki, T., Blumberg, R.S., Nagai, R. and Matsuhashi, N. (2001) Gastroenterology 120, 460–469.
[19] Saubermann, J.L., Wada, K., Zhao, S.P., Nakajima, A., Blumberg, R.S. and Matsuhashi, N. (2001) Gastroenterology 120 (Suppl.), 3752.
[20] Wada, K., Nakajima, A. and Blumberg, R.S. (2001) Trends Mol. Med. 7, 329–331.
[21] MacMillan-Crow, L.A., Crow, J.P., Kerby, J.D., Beckman, J.S. and Thompson, J.A. (1996) Proc. Natl. Acad. Sci. USA 93, 11853–11858.
[22] Shibuya, A., Wada, K., Nakajima, A., Saeki, M., Niwa, H. and Kamisaki, Y. (2002) Jpn. J. Pharmacol. 88 (Suppl.), 122.
[23] Kliewer, S.A., Forman, B.M., Blumberg, B., Ong, E.S., Borgmeyer, U., Mangelsdorf, D.J., Umesono, K. and Evans, R.M. (1994) Proc. Natl. Acad. Sci. USA 91, 7355–7359.