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The role of NF-κB signaling in impaired liver tissue repair in thioacetamide-treated type 1 diabetic rats ☆

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Abstract

Previously we reported that an ordinarily nonlethal dose of thioacetamide (300 mg/kg) causes liver failure and 90% mortality in type 1 diabetic rats, primarily because of inhibited tissue repair. On the other hand, the diabetic rats receiving 30 mg thioacetamide/kg exhibited equal initial liver injury and delayed tissue repair compared to nondiabetic rats receiving 300 mg thioacetamide/kg, resulting in a delay in recovery from that liver injury and survival. These data indicate that impaired tissue repair in diabetes is a dose-dependent function of diabetes. The objective of the present study was to test the hypothesis that disrupted nuclear factor-κB (NF-κB)-regulated cyclin D1 signaling may explain dose-dependent impaired tissue repair in the thioacetamide-treated diabetic rats. Administration of 300 mg thioacetamide/kg to nondiabetic rats led to sustained NF-κB-regulated cyclin D1 signaling, explaining prompt compensatory tissue repair and survival. For the first time, we report that NF-κB–DNA binding is dependent on the dose of thioacetamide in the liver tissue of the diabetic rats. Administration of 300 mg thioacetamide/kg to diabetic rats inhibited NF-κB-regulated cyclin D1 signaling, explaining inhibited tissue repair, liver failure and death, whereas remarkably higher NF-κB–DNA binding but transient down regulation of cyclin D1 expression explains delayed tissue repair in the diabetic rats receiving 30 mg thioacetamide/kg. These data suggest that dose-dependent NF-κB-regulated cyclin D1 signaling explains inhibited versus delayed tissue repair observed in the diabetic rats receiving 300 and 30 mg thioacetamide/kg, respectively.

Keywords: Diabetes; Liver; NF-κB; Thioacetamide; Tissue repair

1. Introduction

Type 1 as well as type 2 diabetes are known to potentiate the hepatotoxicity of numerous structurally and mechanistically diverse hepatotoxicants such as thioacetamide, $CHCl_3$, and CCl_4 (El-Hawari and Plaa, 1983; Hanasono et al., 1975; Sawant et al., 2004). A recent epidemiological study shows that diabetic patients are at higher risk of acute liver failure (El-Serag and Everhart, 2002). Previously, our laboratory reported that exposure of type 1 diabetic rats to an ordinarily nonlethal dose of thioacetamide (300 mg/kg) exhibited higher initial liver injury and 90% mortality, primarily due to inhibited tissue repair (Wang et al., 2000a). It could be argued that inhibited compensatory tissue repair observed in the diabetic rats receiving 300 mg thioacetamide/kg was simply due to CYP2E1-mediated higher initial liver injury (Wang et al., 2000b) and lower number of healthy hepatocytes available to divide. To investigate this possibility, 30 mg thioacetamide/kg was administered to diabetic rats, which exhibited initial liver injury equal to the nondiabetic rats receiving 300 mg thioacetamide/kg (Wang et al., 2000a) (Fig. 1A). In

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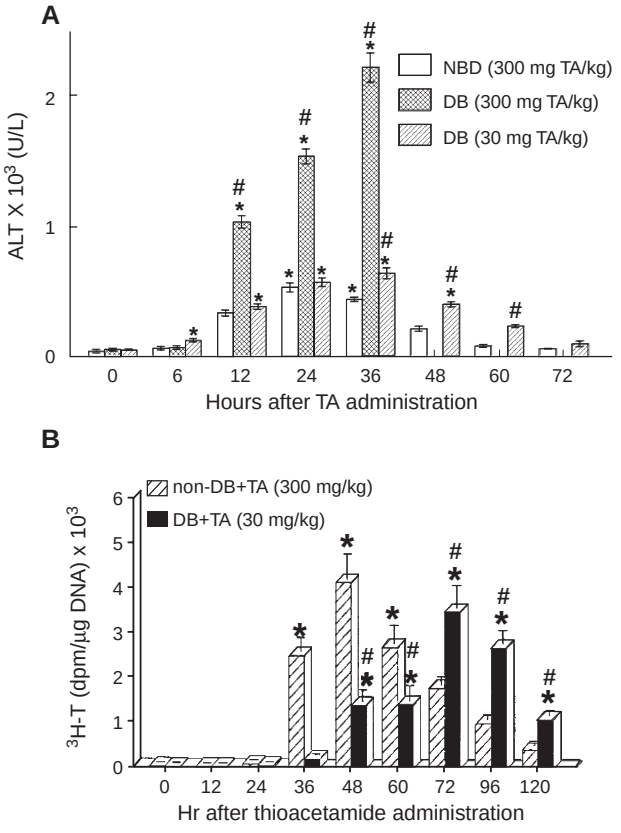


Fig. 1. On day 0, male Sprague–Dawley rats (250 to 300 g) received either a single dose of streptozotocin (60 mg/kg, 0.01 M citrate buffer as vehicle, i.p.) to induce diabetes or citrate buffer (0.01 M, pH 4.3, 1 ml/kg, i.p.) as the nondiabetic control. On day 9, plasma glucose level was measured and animals were considered diabetic if plasma glucose level was ≥250 mg/dl (Mean 390±112 mg/dl). On day 10, diabetic rats were divided into two groups. The first group of diabetic rats (n=4 for all time points except 36 h where n=8 was used to obtain n=4 of surviving animals) was treated with thioacetamide (300 mg/kg, i.p.), while the second group of diabetic rats (n=4) was treated with 30 mg thioacetamide/kg i.p. Citrate buffer receiving control group i.e. nondiabetic group (n=4) received 300 mg thioacetamide/kg. At 0, 6, 12, 24, and 36 h after thioacetamide administration, plasma and liver samples were collected and stored at −20 and −80 °C, respectively. A. Plasma alanine aminotransferase activity over the time course of 0 to 72 h. Diabetic rats receiving 300 mg thioacetamide/kg exhibited higher initial liver injury, which is progressive and irreversible. Even though a 10-fold lower dose of thioacetamide (30 mg thioacetamide/kg) causes equal bioactivation-mediated liver injury in diabetic rats as observed in nondiabetic rats receiving 10-fold higher dose, completely divergent outcomes are evident in the time course of injury and recovery. Liver injury is progressive in the diabetic rats whereas in the nondiabetic rats it decreases promptly indicating complete recovery. B. [^{3}H]-T incorporation from 0 to 120 h after thioacetamide administration. [^{3}H]-T (35 µCi i.p.) was given 2 h before animal sacrifice (Wang et al., 2000a). In the same study described in panel A, stimulation of S-phase was measured as hepatonuclear incorporation of [^{3}H]-T in pulse labeling experiments. Prompt stimulation of S-phase in nondiabetic rats peaking at 48 h is indicative of prompt and robust stimulation of tissue repair leading to prompt recovery from injury as evident in panel A. In contrast, stimulation of S-phase was considerably delayed in the diabetic rats even though they received a 10-fold lower dose of thioacetamide (30 mg/kg), leading to progression of liver injury (Panel A). Although delayed, adequate stimulation of tissue repair does occur, peaking at 72 h in the diabetic rats, permitting complete recovery (Panel B).

spite of equal initial bioactivation-mediated liver injury, diabetic rats receiving 30 mg thioacetamide/kg exhibited delayed S phase DNA synthesis as measured by [^{3}H]-thymidine incorporation (Fig. 1B), resulting in delayed recovery from liver injury and survival. These findings indicated that impairment of tissue repair is due to the diabetic condition and plays a determinant role in the final outcome of the injury, i.e. survival or delayed recovery from the injury (Wang et al., 2001; Wang et al., 2000b). Therefore, any understanding of molecular mechanisms responsible for impaired tissue repair response is of continued clinical interest.

Our previous studies with [^{3}H]-thymidine pulse labeling and proliferating cell nuclear antigen (PCNA) identified the inability of thioacetamide-treated diabetic rat hepatocytes to progress from G_0 to S phase of cell division cycle (Wang et al., 2000a). Numerous studies have confirmed the pivotal role of nuclear transcription factor kappa B (NF-κB) signaling in liver regeneration, especially in the early phase of the cell cycle (Baldwin, 1996; FitzGerald et al., 1995; Perkins, 2000). NF-κB is a crucial factor during liver regeneration after partial hepatectomy to trigger cell cycle progression of hepatocytes (Plumpe et al., 2000). NF-κB controls cell growth and differentiation through transcriptional regulation of cyclin D1 (Guttridge et al., 1999). Cyclin D1 promotes mitogen-independent cell cycle progression in hepatocytes (Albrecht and Hansen, 1999). We hypothesized that a dose-dependent perturbation of NF-κB-regulated cyclin D1 signaling in the thioacetamide-treated diabetic rats may offer an explanation for inhibited versus delayed tissue repair observed in the diabetic rats receiving 300 versus 30 mg thioacetamide/kg, respectively.

In a normal adult liver, NF-κB is retained in the cytoplasm by its endogenous inhibitor, IκBα. Cytokines like tumor necrosis factor-α (TNF-α) stimulate IκB kinase (IKK) to phosphorylate IκBα, releasing NF-κB to translocate into the nucleus (Brown et al., 1995; Rothwarf and Karin, 1999). Another signaling pathway, mitogen activated protein kinase (MAPKs), is also known to trigger NF-κB pathway. In the nucleus, NF-κB binds to cognate DNA sequences and activates transcription of the target genes including cell cycle regulatory cyclin D1. We report here that thioacetamide-treated nondiabetic group exhibited sustained NF-κB–DNA binding and cyclin D1 expression. In contrast, NF-κB-regulated cyclin D1 expression was inhibited in the diabetic rats receiving 300 mg thioacetamide/kg, explaining inhibited tissue repair that leads to progression of injury, liver failure and death in this group. On the other hand, in the diabetic rats receiving a 10-fold lower dose of thioacetamide (30 mg/kg) NF-κB–DNA binding was remarkably stimulated, indicating NF-κB–DNA binding exhibits thioacetamide dose-dependent phenomenon in the diabetic condition. Even though NF-κB–DNA binding was increased in the diabetic rats exposed to 30 mg thioacetamide/kg, delayed cyclin D1 expression explains delayed tissue repair and late recovery of the diabetic rats in this group.

2. Materials and methods

2.1. Induction of diabetes and thioacetamide treatment

Male Sprague–Dawley rats (250 to 300 g) were obtained from our central animal facility. The rats received commercial rodent chow (Teklad rodent diet # 7002, Harlan Teklad, Madison, WI) and water ad libitum and were housed under controlled temperature (21±1 °C), humidity (50±10%), and a 12-h photoperiod. Animal care and use were in accordance with the NIH Guide for the care and use of laboratory animals. On day 0, rats received either a single dose of streptozotocin (60 mg/kg, 0.01 M citrate buffer as vehicle, i.p.) to induce diabetes or citrate buffer (0.01 M, pH 4.3, 1 ml/kg, i.p.) to serve as the nondiabetic control. On day 9, plasma glucose level was measured and animals were considered diabetic if plasma glucose level was ≥200 mg/dl (Mean 390±112 mg/dl). On day 10, diabetic rats were divided into two groups. The first group of diabetic rats was treated with thioacetamide (300 mg/kg, i.p.) [n=4 per time point, except for 36 h time point where n=8 was used in order to obtain adequate number (n=4) of surviving animals], while the second group of diabetic rats (n=4) was treated with 30 mg thioacetamide/kg i.p. The control nondiabetic group treated with citrate buffer (n=4) received 300 mg thioacetamide/kg i.p. At 0, 6, 12, 24, and 36 h after thioacetamide administration, plasma and liver samples were collected and stored at −20 and −80 °C, respectively, until use. For both the diabetic groups receiving 300 as well as 30 mg thioacetamide/kg, one common 0 h control group, i.e. diabetic rats at 0 h was used.

2.2. Lethality study

On day 0, rats were treated with streptozotocin (60 mg/kg i.p.) or 0.01 M citrate buffer (pH 4.3, 1 ml/kg, i.p.). On day 10, diabetic rats were treated with 300 and 30 mg thioacetamide/kg (n=10 per group), while nondiabetic rats were treated with 300 mg thioacetamide/kg (n=10). Rats were observed twice daily for 14 days. Survival/mortality was recorded.

2.3. Plasma alanine aminotransferase

Plasma was separated by heparinization and centrifugation. Plasma alanine aminotransferase was measured as biomarker of liver injury using Sigma kit no. 59 UV.

2.4. TNF-α enzyme linked immunosorbent assay (ELISA)

Plasma TNF-α level was estimated according to manufacturer's protocol (R&D Systems Inc., Minneapolis, MN).

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were isolated from liver tissues at 0, 12, 24 and 36 h after thioacetamide treatment as described previously (Essani et al., 1997). EMSA was performed using gel shift assay system from Promega (Madison, WI) according to the manufacturer's directions by using [^{32}P]. Subsequent to electrophoresis by using 5% non-denaturing polyacrylamide gels at room temperature, gels were dried and exposed to X-Omat film (Eastman Kodak, Rochester, NY) for autoradiography. Cold competition step in EMSA confirmed that the band observed on the gel was NF-κB. Blots were scanned using a GS-800 imaging densitometer (BioRad).

2.6. Western blot analyses

Cytosolic extract was prepared as described previously (Shankar et al., 2003) and 50 μg of total protein was subjected to sodium dodylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. Membranes were blocked in 5% dried milk in 0.1% Tween 20 in 1X tris buffered saline (TBS), and then incubated with the respective primary antibodies [p65, phospho-p65, IKK (Rockland, Gilbertsville, PA), phospho-IKK αβ-Ser 180/181 (United States Biological Inc., Swampscott, MA), total p42/p44, phospho-p42/p44-Thr202/Tyr204, cyclin D1, phospho-IκBα-Ser 32 (Cell Signaling Technology, Beverly, MA), TNF-α (R&D Systems Inc., Minneapolis, MN)] overnight followed by horseradish peroxidase-labeled secondary antibody and then detected by chemiluminescence detection system (Pierce, Rockford, IL). Densitometric analysis was performed by using a GS-800 imaging densitometer (BioRad). Optical density was normalized to GAPDH, as an internal control (Santacruz Biotechnology Inc., Santa Cruz, CA) and expressed as percent optical density of the nondiabetic sample at 0 h.

2.7. Assessment of apoptotic cells

Portions of liver were taken from the left lobes and fixed immediately in 10% phosphate-buffered formalin. The liver tissue samples were processed and then embedded in paraffin. Liver sections (5 μm thickness) were processed for the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using ApoTag kit (Serologicals Co., Norcross, GA) for the evaluation of nuclear morphology over the time course of 0 to 36 h after thioacetamide administration. Three slides per group per time point were stained and the extent of apoptosis was scored under a light microscope using ×400 magnification as described previously (Gujral et al., 2001; Mangipudy and Mehendale, 1998; Mangipudy et al., 1998). The morphometric analysis of apoptosis was done as a blinded study.

2.8. Statistical analysis

Data were expressed as means±S.E.M. Statistical differences were determined by one-way analysis of variance followed by Tukey's HSD and Duncan's multiple range tests to determine which means were significantly different from

each other or from controls using SPSS software (SPSS Inc., Chicago, IL). Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Lethality study

Toxicity caused by thioacetamide was increased substantially in the diabetic rats receiving 300 mg thioacetamide/kg as indicated by 90% mortality as compared to 0% mortality observed in the nondiabetic group receiving the same dose of thioacetamide. Death occurred between 30 and 60 h after thioacetamide administration to diabetic rats. No mortality occurred in the diabetic group receiving 30 mg thioacetamide/kg.

3.2. Plasma transaminase level

Plasma alanine aminotransferase level was measured to evaluate liver injury (Fig. 1A). In the nondiabetic group alanine aminotransferase activity increased at 24 h after thioacetamide administration and then declined gradually towards normal. In contrast, in the diabetic rats receiving 300 mg thioacetamide/kg liver injury was progressive and irreversible from 12 h onward as indicated by dramatically elevated plasma alanine aminotransferase. Equal amount of injury (plasma alanine aminotransferase) was observed up to 24 h in the diabetic rats receiving 30 mg thioacetamide/kg and the nondiabetic rats receiving 300 mg thioacetamide/kg dose. These results are consistent with the earlier published report (Wang et al., 2000a).

3.3. Plasma TNF-α

Diabetes did not change the basal level (0 h) of plasma TNF-α, measured by ELISA (Fig. 2A). In the thioacetamide-treated (300 mg/kg) nondiabetic group, plasma TNF-α level was elevated at 24 h and remained high at 36 h after thioacetamide treatment. Diabetic rats receiving the same dose of thioacetamide exhibited the highest TNF-α level (~3 to 4-fold) as early as 6 h, which remained ~2-fold higher until 12 h. At 24 h after thioacetamide treatment, TNF-α titer was equal in diabetic and nondiabetic rats (Fig. 2A). Diabetic rats receiving 30 mg thioacetamide/kg exhibited a maximum increase in plasma TNF-α level at 6 h, which declined thereafter to the normal plasma titer at 24 and 36 h after thioacetamide administration (Fig. 2A). TNF-α plasma levels were measured in time-matched solvent (saline) control. However, solvent (saline) control did not induce TNF-α, suggesting that observed changes in TNF-α plasma levels were due to thioacetamide administration (data not shown in the interest of simplifying Fig. 2A).

In contrast to plasma TNF-α level, diabetes increased hepatic TNF-α level as measured by immunoblotting (Fig. 2B). In the nondiabetic rats receiving a dose of 300 mg thioacetamide/kg, hepatic TNF-α was down regulated until 12 h, but upregulated at 24 and 36 h after thioacetamide treatment. On the other hand, TNF-α increased by diabetes was further increased in the diabetic rats receiving 300 mg thioacetamide/kg until 24 h, declining at 36 h after thioacetamide-treatment. A 10-fold lower dose of thioacetamide (30 mg/kg) resulted in decreased hepatic expression of TNF-α throughout the time course of 6 to 36 h after thioacetamide administration. Densitometric data were normalized to GAPDH, an internal control (Fig. 2 C).

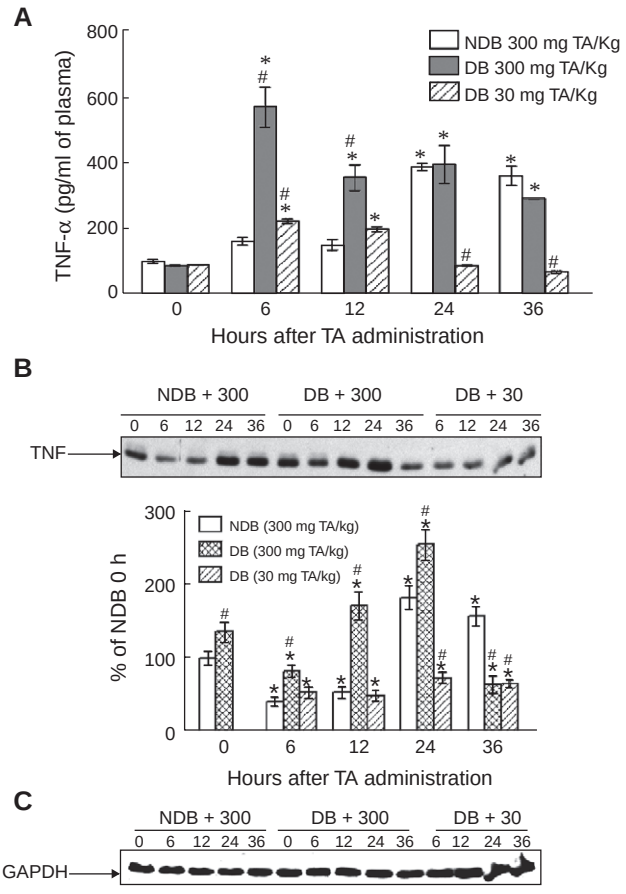


Fig. 2. TNF-α level. Experimental protocol is as described for in Fig. 1. A. Plasma TNF-α levels were measured over the time course using a rat-specific ELISA after a single administration of 300 mg thioacetamide/kg to nondiabetic rats and either 300 or 30 mg thioacetamide/kg to diabetic rats. TNF-α plasma levels were measured in time-matched solvent (saline) control. However, solvent (saline) control did not induce TNF-α, suggesting that observed changes in TNF-α serum levels were due to thioacetamide administration (data not shown in the interest of simplifying A). B. Hepatic TNF-α levels were measured by immunoblotting. This blot is representative of four independent experiments. Densitometric quantitative analysis is provided. Values are expressed as mean±S.E.M. ($n=4$). *Significantly different from 0 h value in the corresponding group. #Significantly different from the nondiabetic group receiving 300 mg thioacetamide/kg at the corresponding time point. C. GAPDH was used as internal control.

3.4. NF-κB–DNA binding

Neither dose of thioacetamide affected NF-κB–DNA at 3 h in the nondiabetic and diabetic rats. In the 300 mg

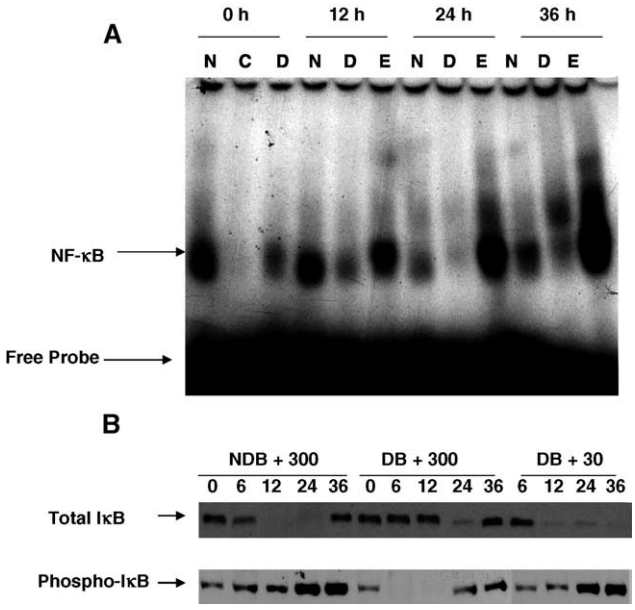


Fig. 3. A. NF-κB–DNA binding. Experimental protocol is as described in Fig. 1. Diabetic and nondiabetic rats were treated with thioacetamide and NF-κB–DNA binding was measured by electrophoretic mobility shift assay (EMSA) as described in experimental procedures. N = nondiabetic rats treated with 300 mg thioacetamide/kg, C = Cold competition assay was performed to confirm NF-κB band, D = diabetic rats treated with 300 mg thioacetamide/kg, and E = diabetic rats receiving 30 mg thioacetamide/kg. This blot is representative of four independent experiments. B. Expression of total and phosphorylated form of IκB. Blots are representative of four independent experiments. GAPDH was used as an internal control.

thioacetamide/kg-treated nondiabetic rats, NF-κB–DNA binding was unchanged at 12 h, decreased at 24 h, returning to normal at 36 h after thioacetamide treatment (Fig. 3A). In contrast, NF-κB–DNA binding was remarkably suppressed in the diabetic rats receiving the same dose of thioacetamide (300 mg/kg) over the time course of 0 to 36 h (Fig. 3A). On the other hand, diabetic rats receiving a 10-fold lower dose of thioacetamide (30 mg/kg) exhibited higher NF-κB–DNA binding over the time course of 0 to 36 h after thioacetamide administration (Fig. 3A).

3.5. Phosphorylation of IκBα and subsequent phosphorylation of p65 subunit of NF-κB

Phosphorylation of IκBα at Ser32 is essential for the release and action of active NF-κB (Beg and Baldwin, 1993). Therefore, the IκBα phosphorylation was assessed by using monoclonal antibody specific for Ser32 site of IκBα protein (Fig. 3B). Diabetic condition showed a decrease in IκBα phosphorylation. Because of 300 mg/kg thioacetamide treatment to the nondiabetic rats, IκBα phosphorylation was increased over the time course of 0 to 36 h after thioacetamide administration. Phosphorylation of IκBα was inhibited by ~25% in the diabetic rats at basal level, which was further inhibited until 12 h after 300 mg thioacetamide/kg treatment. In this group, phospho-IκB returned to normal by 24 h and increased at 36 h after thioacetamide administration. On the other hand, in the diabetic rats receiving 30 mg thioacetamide/kg phosphorylation of I κBα was unchanged until 12 h, increasing substantially at 24 and 36 h (~5-fold as compared to diabetic rats at 0 h) after thioacetamide administration.

Diabetes itself did not change the expression of total p65 subunit of NF-κB (Fig. 4A). In the nondiabetic rats receiving 300 mg thioacetamide/kg, expression of total p65 was lower at 6 h, which then returned to normal by 12 h, and increased thereafter. In the diabetic rats receiving 300 mg thioacetamide/kg, expression of total p65 was unaffected over the time course of 0 to 36 h after thioacetamide administration. On the other hand, diabetic rats receiving 30 mg thioacetamide/kg exhibited increased expression of total p65 at 24 and 36 h after thioacetamide administration.

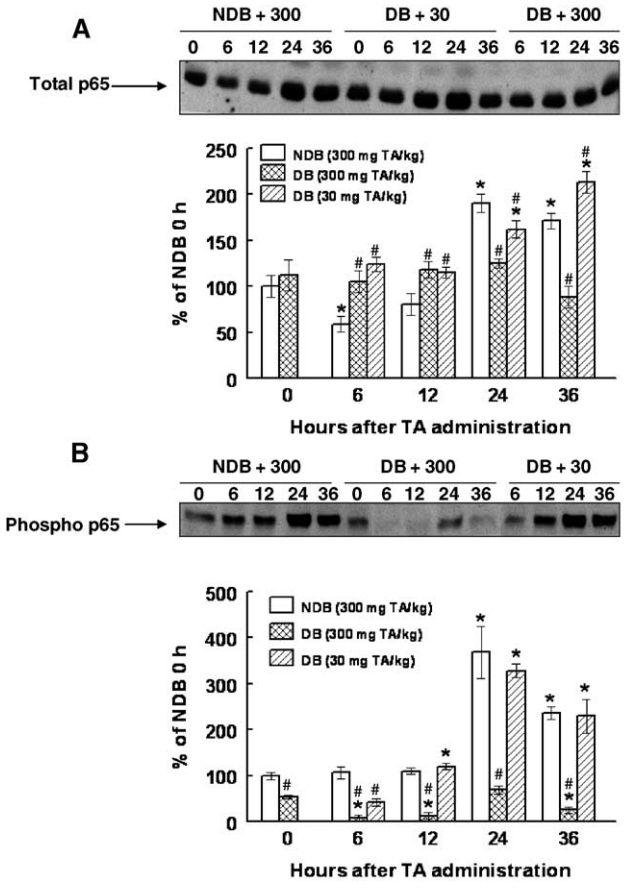


Fig. 4. Expression of p65 subunit of NF-κB. A. Representative immunoblot of total p65. B. Representative immunoblot of phosphorylated form of p65. Experimental protocol is as described in Fig. 1. From the liver samples of the nondiabetic rats treated with 300 mg thioacetamide/kg and the diabetic rats treated with 300 and 30 mg thioacetamide/kg, cytoplasmic extract was prepared as described in experimental procedure and followed by immunoblotting. Blots are representative of four independent experiments. Densitometric quantitative analysis is provided. Values are expressed as mean ± S.E.M. (n=4). *Significantly different from 0 h value in the corresponding group. #Significantly different from the nondiabetic group receiving 300 mg thioacetamide/kg at the corresponding time point. Data were normalized by using GAPDH as an internal control.

Expression of phosphorylated p65 was inhibited by 50% in the diabetic rats at basal level (0 h) (Fig. 4B). Administration of 300 mg thioacetamide/kg to nondiabetic rats did not change the expression of phospho-p65 until 12 h, which was increased at 24 (~3.7-fold) and 36 h (~2.4-fold) after thioacetamide treatment. In contrast, in the diabetic rats receiving the same dose of thioacetamide (300 mg thioacetamide/kg), expression of phospho-p65 was substantially decreased over the time course after thioacetamide treatment with the exception of 24 h time point. On the other hand, diabetic rats receiving 30 mg thioacetamide/kg exhibited increased phosphorylation of p65 subunit of NF-κB at 12 (~2-fold), 24 (~6.1-fold) and 36 h (~4-fold) as compared to the 0 h basal diabetic level.

3.6. Expression of IKK

Neither diabetic condition nor thioacetamide treatment changed the expression of IKK in any of the groups until 12 h (Fig. 5A). A robust increase in IKK expression was observed in all the groups at 24 h after thioacetamide administration. However, at 36 h after thioacetamide treatment, IKK expression remained robust only in the diabetic rats receiving 30 mg thioacetamide/kg and non-diabetic rats receiving 300 mg thioacetamide/kg.

Catalytic activity of IKK resides in the three tightly associated IKK subunits: IKK-α and IKK-β serve as the catalytic subunits of IKK, whereas IKK-γ serves as regulatory subunit (Delhase et al., 1999; Verma et al., 2004). Activation of IKK depends on phosphorylation at the specific site of Ser 180 of IKK-α and Ser 181 of IKK-β causing a conformational change that results in IKK activation. Therefore, the phosphorylated form of IKK protein was estimated by using an antibody that specifically detects phosphorylated sites at Ser 180 (IKK-α) and Ser 181 on (IKK-β) respectively. IKK phosphorylation did not differ between diabetic and nondiabetic rats (Fig. 5B). In the nondiabetic rats, phosphorylation of IKK was unaffected until 12 h, robustly increased at 24 (~3.4-fold), and 36 h (~5.6-fold) after 300 mg thioacetamide/kg treatment (Fig. 5B). In contrast, in the diabetic rats receiving the same dose of thioacetamide (300 mg/kg), IKK phosphorylation was inhibited at 12 h, returning to normal at 24 and 36 h after thioacetamide administration. It should be noted that phospho-IKK expression was significantly lower in the 300 mg thioacetamide/kg treated diabetic rats as compared to the thioacetamide-treated nondiabetic rats (Fig. 5B). On the other hand, diabetic rats receiving 30 mg thioacetamide/kg exhibited increased levels of phospho-IKK protein at 24 (~4-fold) and 36 h (~6.6-fold) after thioacetamide administration.

3.7. Expression of MAPKs and cyclin D1

Diabetes decreased the expression of total MAPKs at the basal level (Fig. 6A). Thioacetamide-treated nondiabetic group exhibited sustained expression of total MAPKs over the time course of 0 to 36 h after thioacetamide administration. In contrast, diabetic rats receiving 300 mg thioacetamide/kg exhibited markedly decreased expression of MAPKs over the time course of 0 to 36 h (with the exception of 24 h) after thioacetamide administration. On the other hand, in the diabetic rats receiving 30 mg thioacetamide/kg, total MAPKs expression was down regulated at 6 and 12 h, increasing at 24 and 36 h after thioacetamide treatment. In the nondiabetic rats, phosphorylation of MAPKs (ERK1 and ERK2) was robust at 12 and 24 h, declining at 36 h after 300 mg thioacetamide/kg treatment (Fig. 6B). In contrast, ERK1 and ERK2 phosphorylation was

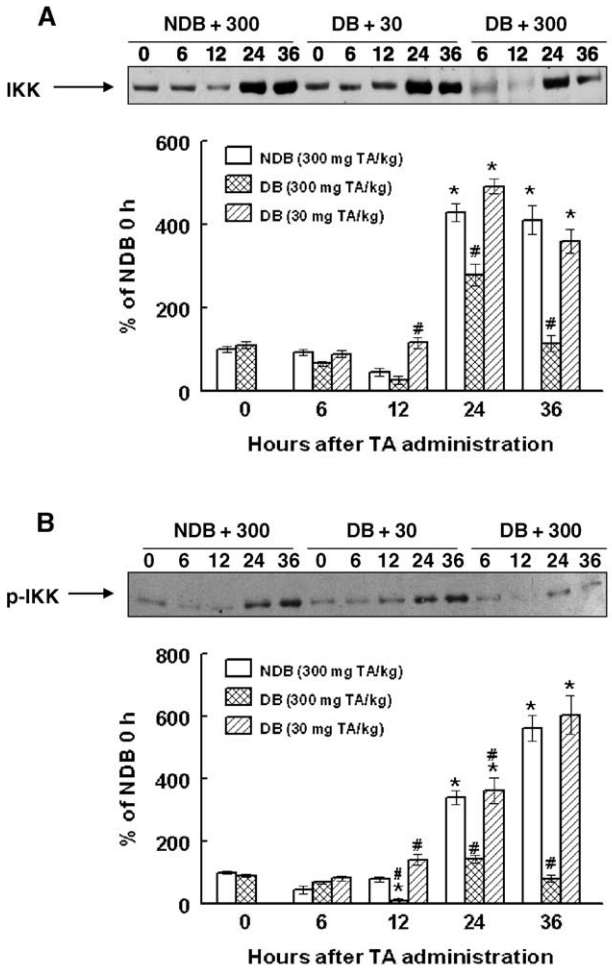


Fig. 5. Expression of IKK. A. Representative immunoblot of total IKK. B. Representative immunoblot of phosphorylated form of IKK. Experimental protocol is as described in Fig. 1. From the liver samples of the nondiabetic rats treated with 300 mg thioacetamide/kg and the diabetic rats treated with 300 and 30 mg thioacetamide/kg, cytoplasmic extract was prepared as described in experimental procedure and followed by immunoblotting. Blots are representative of four independent experiments. Densitometric quantitative analysis is provided. Values are expressed as mean±S.E.M. (n=4). *Significantly different from 0 h value in the corresponding group. #Significantly different from the nondiabetic group receiving 300 mg thioacetamide/kg at the corresponding time point. Data were normalized by using GAPDH as an internal control.

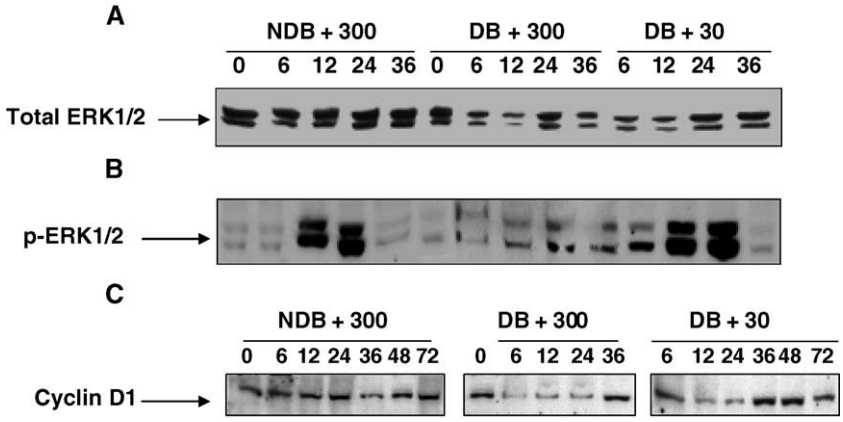


Fig. 6. Expression of MAPKs and cyclin D1. A. Representative immunoblot of total MAPKs (ERK1 and ERK2). B. Representative immunoblot of phosphorylated form of MAPKs. C. Representative immunoblot of cyclin D1. Blots are representative of four independent experiments. GAPDH was used as an internal control.

inhibited in the diabetic rats exposed to the same dose of thioacetamide over the entire time course of 0 to 36 h (Fig. 6B). On the other hand, phosphorylation of ERK1 and ERK2 was higher in the diabetic rats receiving 30 mg thioacetamide/kg as early as 6 h, markedly increasing further at 12 h and 24 h.

No significant difference was observed in the cyclin D1 protein expression by diabetic state alone (Fig. 6C). Nondiabetic rats receiving 300 mg thioacetamide/kg exhibited sustained expression of cyclin D1 protein over the entire time course of 0 to 72 h after thioacetamide administration. In contrast, diabetic rats receiving the same dose of thioacetamide (300 mg/kg) exhibited markedly inhibited cyclin D1 protein expression over the time course of 12 to 36 h (~80%) after thioacetamide administration. On the other hand, diabetic rats receiving 30 mg thioacetamide/kg exhibited initial decrease in cyclin D1 expression at initial time points until 24 h, returning to normal by 36 h, and thereafter remaining sustained until 72 h, suggesting a delay in the expression of cyclin D1 in these rats.

3.8. Apoptosis in thioacetamide-treated diabetic rat liver

Diabetes did not affect apoptosis (Data not shown). After thioacetamide treatment, approximately 2% of the hepatocytes were apoptotic at 24 h after 300 mg thioacetamide/kg in the rats regardless of diabetes. On the other hand, diabetic rats exposed to 30 mg thioacetamide/kg exhibited significantly lower apoptosis at 24 h (less than 0.2%) than the thioacetamide-treated nondiabetic rats receiving 300 mg thioacetamide/kg.

4. Discussion

Numerous studies have shown that tissue repair plays a determinant role in the outcome of the injury (survival/death) after toxicant challenge (Horn et al., 1999; Mangipudy et al., 1996; Mehendale, 1991, 2005; Ramaiah et al., 2000). However, the mystery behind how the hepatocytes orchestrate various molecular signaling events after toxicant challenge to accomplish tissue repair remains largely unsolved. Most of the studies designed to understand the signaling pathways involved in the liver regeneration use either partial hepatectomy or in vitro models, which lack the dynamic interaction between ongoing liver injury and ongoing tissue repair. Therefore, in the present study, we employed a toxicant-induced liver injury model to simulate predisposition to drug-induced toxicities in diabetes.

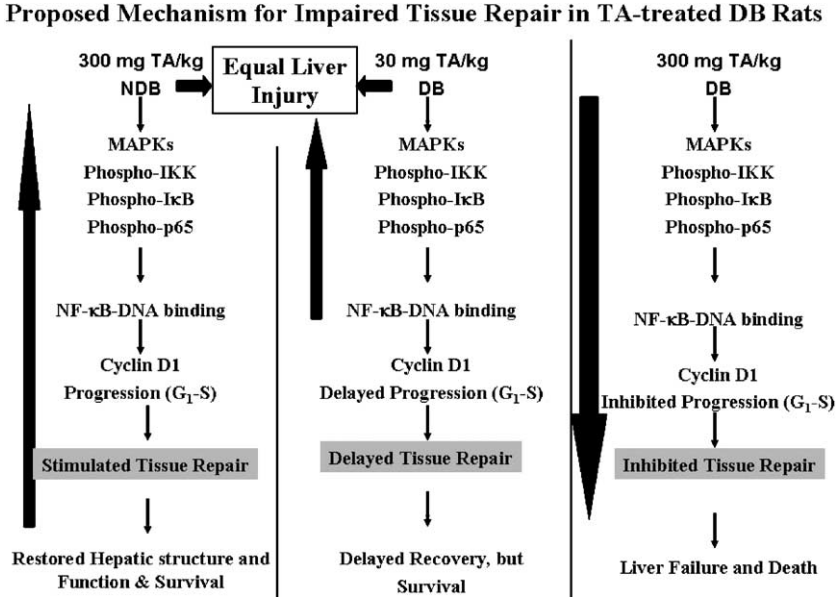
NF-κB has been shown to regulate the expression of a number of exogenous and endogenous growth factors that are known to promote cellular proliferation (Gugasyan et al., 2000). Multiple NF-κB binding sites have been reported in the promoter region of cyclin D1, an important cell cycle regulator that, upon association with cdk4 and cdk6, promotes the progression of cells from G_0/G_1 to S phase (Guttridge et al., 1999). Using diploid fibroblasts, it has been demonstrated that NF-κB is required to induce cyclin D1 expression and retinoblastoma protein (pRb) hyperphosphorylation and promote G_1 to S progression (Guttridge et al., 1999). Rapid NF-κB–DNA binding has been demonstrated at the early stage of the liver regeneration in the partial hepatectomy model (FitzGerald et al., 1995), whereas inhibition of NF-κB activation during liver regeneration in mice led to significant decrease in DNA replication (Plumpe et al., 2000). Collectively, these data suggest that NF-κB–DNA binding plays a crucial role in the cell cycle progression. Therefore, in the present study NF-κB signaling pathway was investigated to understand the molecular mechanisms that may explain inhibited versus delayed tissue repair in the diabetic rats receiving 300 versus 30 mg thioacetamide/kg, respectively. For the first time, we report that the adverse effect of thioacetamide on NF-κB–DNA binding is dose-dependent. In the diabetic rat liver, 300 mg/kg dose of thioacetamide was found to inhibit NF-κB–DNA binding over the time course of 0 to 36 h, whereas a 10-

fold lower dose of thioacetamide remarkably stimulated the binding of NF-κB to nuclear DNA at 24 and 36 h after thioacetamide administration (Fig. 3A). Upstream signaling to NF-κB–DNA binding was investigated to explain the dose-dependent effect of thioacetamide on NF-κB–DNA binding in diabetic rat liver.

NF-κB activation requires TNF-α. It is also known to be pro-proliferative through stimulation of immediate early genes to expedite the priming (G_0 to G_1) phase of the cell cycle (Akerman et al., 1992; Bruccoleri et al., 1997). Pretreatment with antibodies to TNF-α has been shown to inhibit [^{3}H]-T incorporation into DNA as well as expression of PCNA by both hepatocytes and liver nonparenchymal cells, indicating that TNF-α positively modulates liver regeneration (Akerman et al., 1992). It is clear from our results that priming signaling via TNF-α is higher in the diabetic rats receiving thioacetamide. Therefore, we examined the regulatory mechanisms involved in NF-κB activation: (1) phosphorylation of IκB; and (2) phosphorylation of p65 subunit NF-κB. These two mechanisms can also be regulated by MAPK signaling pathway, independent of TNF-α activation.

Sustained MAPKs activation (Fig. 6A and B) in the thioacetamide-treated nondiabetic rats leads to phosphorylation of IKK (Fig. 5) and IκB (Fig. 3B), resulting in subsequent translocation of p65 subunit of NF-κB (Fig. 4) (Scheme 1). It is surprising that in the thioacetamide-treated nondiabetic group, even though phosphorylation of p65 subunit was remarkably higher at 24 and 36 h (Fig. 4B), NF-κB–DNA binding was not increased at the respective time points. One explanation for this might be that transcriptional efficiency of NF-κB can also be modified by its interactions with a plethora of transcriptional coactivators like CREB binding protein (CBP) and p300 to stimulate transcriptional activation of target genes (Zhong et al., 1998). Therefore, modification in the interaction between p65 and one of the coactivators might have compensated for increased phosphorylation of p65. It should be noted that sustained NF-κB–DNA binding was observed over the time course of 0 to 36 h after thioacetamide treatment in the nondiabetic rats. In the diabetic rats receiving higher dose of thioacetamide (300 mg /kg), inhibited phosphorylation of MAPKs (Fig. 6B) resulted in decreased phosphorylation of IKK (Fig. 5B), IκB (Fig. 3B), and p65 (Fig. 4B), explaining inhibited NF-κB–DNA binding (Fig. 3A) observed in these rats. In contrast, hefty phosphorylation of MAPKs in the diabetic rats receiving a 10-fold lower dose of thioacetamide (30 mg/kg) resulted in sustained phosphorylation of IKK (Fig. 5), IκBα (Fig. 3B), and p65 subunit of NF-κB after thioacetamide administration. This ~6-fold higher phosphorylation of p65 may explain remarkably higher NF-κB–DNA binding in these rats (Fig. 3A).

Because TNF-α is pro-apoptotic and NF-κB is anti-apoptotic, apoptosis was assessed by TUNEL assay in the thioacetamide-treated nondiabetic and diabetic rats over the time course of 0 to 36 h after thioacetamide administration. Only a marginal increase in apoptosis was observed and turned out to be not a factor of any consequence for cell death



Scheme 1. Proposed mechanism for inhibited and delayed tissue repair in the diabetic rats treated with 300 and 30 mg thioacetamide/kg, respectively. In the nondiabetic rats, administration of 300 mg thioacetamide/kg leads to stimulation of NF-κB-mediated priming (G_0 to G_1) and cyclin D1-mediated progressive (G_1 to S) phase of the cell division cycle, resulting in robust tissue repair and quicker recovery from liver injury. By contrast, the diabetic rats receiving the same dose of thioacetamide (300 mg/kg) exhibited inhibited priming and progressive phases of the cell cycle, evidenced by inhibited NF-κB and cyclin D1 signaling mechanisms, which led to progression of liver injury, ultimately resulting in liver failure and animal death. On the other hand, in the diabetic rats receiving a 10-fold lower dose of thioacetamide (30 mg/kg), stimulated NF-κB-mediated priming (G_0 to G_1) but down regulated cyclin D1-mediated progressive (G_1 to S) phase of the cell cycle may explain delayed tissue repair and delayed recovery from liver injury.

(data not shown). Evidently, the thioacetamide-treated diabetic rats, the contribution of apoptotic cell death is very minimal. It is known that that diabetic rats receiving 300 mg thioacetamide/kg undergo necrotic cell death (Wang et al., 2000a). Since apoptosis is not a major factor, this investigation was focused on the NF-κB mediated transcription of proliferative genes. NF-κB mainly exhibits its cell proliferation activity via cyclin D1 expression (Guttridge et al., 1999; Hinz et al., 1999; Takebayashi et al., 2003). Cyclin D1 is widely believed to regulate progression through G_1 phase of the cell cycle, and numerous studies have shown that cyclin D1 is induced during hepatocyte proliferation in culture and in vivo (Albrecht et al., 1993; Cressman et al., 1996; Loyer et al., 1996). Sustained NF-κB-regulated cyclin D1 signaling (Fig. 6C) was observed in nondiabetic rats receiving 300 mg thioacetamide/kg, resulting in timely and adequate compensatory tissue repair in these rats (Scheme 1). In contrast, inhibited NF-κB–DNA binding in diabetic rats receiving the same dose of thioacetamide (300 mg/kg) resulted in severe inhibition of cyclin D1 expression (Fig. 6C), explaining inhibited tissue repair (Scheme 1). On the other hand, in diabetic rats receiving a 10- fold lower dose of thioacetamide (30 mg/kg), even though markedly higher NF-κB–DNA binding was observed, cyclin D1 expression was delayed. Collectively, these data may explain delayed tissue repair observed in diabetic rats receiving 30 mg thioacetamide/kg (Scheme 1). Further interventional studies by using MAPK and NF-κB inhibitors are necessary to confirm the cause effect mechanistic relationship between MAPKs-regulated NF-κB signaling and tissue repair.

The exact mechanism underlying the inhibition of S phase of the cell division in diabetes is not known. Several possibilities exist. One possibility is insulin deficiency. It is known that insulin acts as a complete mitogen, which stimulates molecular signaling, leading to DNA transcription and replication (Stralfors, 1997). Administration of insulin enhanced liver regeneration in rats (Johnston et al., 1986). However, in a recent study, inhibition of compensatory tissue repair in type 2 normoinsulinimic DB rats has been reported (Sawant et al., 2004), suggesting that even in the presence of normal insulin levels compensatory cell division stimulated by thioacetamide is inhibited. Furthermore, insulin and glucagon therapy neither enhanced hepatic synthetic function such as synthesis of prothrombin and alpha-fetoprotein nor did it stimulate hepatic regeneration in patients with fulminant hepatic failure (Harrison et al., 1990). Therefore, factors other than insulin appear to play a role in enhanced hepatotoxicity of thioacetamide in diabetic rats.

Second possibility is hyperglycemia. High glucose inhibits S phase stimulation in cell culture models (Dajani et al., 1994; Rao et al., 1999). Therefore, the hyperglycemia in diabetics might inhibit liver cell proliferation and tissue repair by formation of advanced glycation end (AGE) products. However, in glucose loading experiments it has been reported that S phase stimulation and cell division are inhibited in the presence of normoglycemia and normal insulin in thioacetamide (300 mg/kg) challenged SD rats (Chanda and Mehendale, 1995). Therefore, the role of hyperglycemia remains to be investigated to explain higher sensitivity of hepatotoxicants in diabetic rats.

Third possibility is altered energy status in diabetic condition. A significant decrease in hepatic ATP levels was observed in diabetic rats (Johnston et al., 1986). Ozawa and his colleagues showed that in alloxan-treated diabetic rats, decreased DNA synthesis in the liver after partial hepatectomy was in proportion to the decreased energy status of the remnant liver due to suppressed mitochondrial phosphorylation following insulin deficiency (Ozawa et al., 1981). A significant decrease in hepatic ATP levels has been reported in the diabetic animals after partial hepatectomy (Johnston et al., 1986). Inhibition of ATP synthesis and other essential synthetic pathways lead to impaired cell cycle progression since the macromolecules needed for cell division are not synthesized. Administration of ATP (100 mg/kg, sc) affords protection from chlordecone-amplified CCl_4 hepatotoxicity of an otherwise 90% lethal combination that leads to dramatic depletion of ATP (Soni and Mehendale, 1994). Therefore, depletion of cellular energy in type 1 diabetes might down regulate the cell cycle events, ultimately leading to impaired tissue repair in diabetic state. Nonetheless, our studies shed considerable light on the role of NF-κB signaling in impaired tissue repair in thioacetamide-treated type 1 diabetic rats.

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