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Journal of Nutritional Biochemistry 15 (2004) 342–349

THE JOURNAL OF Nutritional Biochemistry

Caffeine suppresses the expression of the Bcl-2 mRNA in BeWo cell culture and rat placenta

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Received 14 September 2003; received in revised form 11 November 2003; accepted 27 December 2003

Abstract

Chronic caffeine exposure during pregnancy has an effect on fetal growth; however, the adverse effects of caffeine on embryogenesis are not well understood and controversial. We used cDNA microarray technology to determine whether caffeine alters gene expressions in a human cytotrophoblast–like cell line, BeWo. We found that the expression of the B-cell CLL/lymphoma 2 (Bcl-2) gene in BeWo cells was down-regulated by caffeine, suggesting that chronic exposure during the gestational period could exert an influence on embryogenesis. We then focused on the Bcl-2- and Bcl-2–associated X protein gene, Bax, to study the responsive gene expression in BeWo cells as well as placentas of pregnant rats fed a diet supplemented with caffeine (2 mg/100 g body weight) during gestation, and analyzed the gene expressions using LightCycler-based quantitative real-time polymerase chain reaction assays. We found a significantly decreased level of Bcl-2 mRNA expression, which demonstrated the influence of caffeine on placental function.

Keywords: Caffeine; Low birth weight; Fetal growth retardation; Bcl-2; Microarray technology; LightCycler system

1. Introduction

Caffeinated products such as coffee, tea, and cola beverages are widely and routinely consumed by women in their child-bearing years. Interestingly, caffeine consumption by pregnant women has been found to be greater among those with fewer years of formal education [1]. Excessive caffeine intake during pregnancy has been associated as a risk factor for intrauterine growth retardation (IUGR) and low birth weight (LBW) [1–7], as well as for infertility [8], and while it has also been shown to double the risk of spontaneous abortion [9,10]. In contrast, other studies have reported that caffeine exposure was not associated with adverse pregnancy outcomes [11–14].

It is possible that IUGR and LBW are associated with an increased risk of hypertension and coronary heart disease development in adulthood in both men and women. These diseases may be initiated by an inadequate supply of nutrients and/or oxygen *in utero*, with a subsequent effect during life and even on life span [15–17].

Placental metabolic functions are particularly important in early pregnancy to provide nutrition for the developing offspring and the placenta itself [18]. The placenta may also be an important determinant of maternal imprinting events through the control of nutrients to the fetus, as well as hormonal interchanges between fetal and maternal tissues [17].

We have been investigating the role of caffeine on placental gene expression responses in the human cytotrophoblast–like cell line BeWo [19] using cDNA microarrays, which has been useful to observe the temporal program of transcription that underlies these responses. A total of 69 genes, along with angiotensin II type 2 (AT_2) receptor, B-cell CLL/lymphoma 2 (Bcl-2), CD1C antigen, cytokeratin 20, and the gene product KIAA0429, have been found to have a response to caffeine [20]. We also demonstrated that

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0955-2863/04/$ – see front matter
doi:10.1016/j.jnutbio.2003.12.007

the increased expression of AT_2 receptor mRNA in an animal model was due to caffeine exposure during gestation and might impair the balance of blood flow in the placental bed, resulting in IUGR and LBW [21].

Recently, it has been shown that apoptosis is important in many aspects of reproduction and is identified in trophoblasts of the placenta, and is also believed to be physiologically important for normal placental growth and development [22,23]. Furthermore, aberrant placental apoptosis may influence placental function, resulting in complicated pregnancies, as increased apoptosis has been reported in trophoblasts of the placenta with IUGR [24–26]. It has also been demonstrated that the anti-apoptosis regulator Bcl-2 plays an important role in preventing apoptosis of trophoblasts in the human placenta, and high levels of Bcl-2 protein have been shown to be expressed in syncytiotrophoblasts in normal term placentas [27]. On the other hand, Bcl-2 protein expression in syncytiotrophoblasts in IUGR placentas was found to be much less abundant than that in those from normal term placentas [28]. We conducted the present study to determine the effect of caffeine on the expression of the Bcl-2 gene in BeWo cells, as well as the placentas of pregnant rats fed a diet supplemented with caffeine, using a LightCycler-based quantitative real-time polymerase chain reaction method [21,29]. We then expanded the study to investigate the mRNA expression of Bax, an apoptosis promoter, as well as the influence of caffeine on placental function and IUGR.

2. Methods and materials

2.1. Cell culture

A BeWo cell line (CCL-98, American Type Culture Collection, Rockville, MD) with a population of 4×10^5 cells/mL was cultured without or with caffeine at 1.0 mmol/L in Ham's F12-medium containing 10% heat-inactivated fetal bovine serum.

2.2. Tissue preparation

Upon fertilization (day 1), pregnant rats were randomly divided into two groups, with those in group 1 fed a 20% protein diet and those in group 2 given the same 20% protein diet supplemented with caffeine (2 mg/100 g body weight) until day 20 of gestation [30]. On day 20, the placentas were removed by cesarean section. The present study was conducted in accordance with the guidelines for experimental animals of Nihon University School of Dentistry at Matsudo, Japan (ECA-00-0038).

2.3. Total RNA preparation

The cultured cells and frozen placentas were homogenized with a phenol-guanidine isothiocyanate mono-phasic solution, Trizol Reagent (Gibco BRL, Life Technologies, Grand Island, NY), using Fast RNA tubes with matrix (BIO 101, Inc., Vista, CA) in a high-speed homogenizer (Savant Instruments, Farmingdale, NY), and total RNA was isolated by the single-step RNA isolation method developed by Chomczynski and Sacchi [31]. The RNA was then cleaned using traditional phenol/chloroform extraction and precipitation with isopropanol at −20°C, and dissolved in diethylpyrocarbonate–treated distilled H_2O.

2.4. cDNA synthesis

Total RNA was reverse transcribed to cDNA by incubating with oligo $d(T)_{16}$ and Moloney murine leukemia virus reverse transcriptase using a GeneAmp RNA polymerase chain reaction (PCR) kit (Perkin-Elmer, Branchburg, NJ) in a programmable thermal cycler (GeneAmp PCR System 9600, Perkin-Elmer, Norwalk, CT).

2.5. Real-time PCR detection of Bcl-2 and Bax using LightCycler SYBR I technology

The LightCycler PCR and real-time detection system (Roche Diagnostics, Mannheim, Germany) was used for amplification and quantification as previously described [21]. Appropriately diluted cDNA was added to the master mixture from a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics). To set up a quantitative approach for target gene transcripts, we first determined the optimal primer (0.5 μmol/L) and $MgCl_2$ (5 mmol/L) concentrations to obtain amplicon quantity and primer-dimer formation.

The following oligonucleotide primers were designed using oligo-primer analysis software (DNASIS, Hitachi Software Engineering, Tokyo, Japan) based on the GenBank sequence data. The primer sequences used were 5′-TCC AAA ATC AAG TGG GGC GA-3′ and 5′-AGT AGA GGC AGG GAT GAT GT-3′ corresponding to cDNA residues 322–708 in human glyceraldehyde-3-phosphate dehydrogenase (*GAPD*, termed HumGAPD in this study; accession no. NM-002046), which were selected as a positive control for each sample that consisted of the constantly expressed housekeeping gene, 5′-TGT GGC CTT CTT TGA GTT CG-3′ and 5′-ATT TGT TTG GGG CAG GCA TG-3′, which corresponded to cDNA residues 472–777 in the *Homo sapiens* B-cell CLL/lymphoma 2 (BCL2, termed HumBcl-2 in this study; GenBank accession no. NM-000633), and 5′-TAA CAT GGA GCT GCA GAG GA-3′ and 5′-GCT GGC AAA GTA GAA AAG GG-3′, which corresponded to cDNA residues 216 to 354 in the *Homo sapiens* BCL2-associated X protein (*BAX*, HumBax in this study; NM-004324).

Amplification of HumBcl-2 was performed using a LightCycler rotor for 38 cycles programmed with a 4-segment cycle procedure composed of 95°C for 15 seconds, 55°C for 5 seconds, 72°C for 16 seconds, and 85°C for 1

second. The double-stranded PCR product was measured at 85°C by detection of fluorescence associated with the binding of DNA double-strand specific SYBR Green I dye. After amplification, a melting curve analysis was done by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 65°C, and then heating it again at 20°C/s to 95°C with fluorescence collection at 0.1°C/s intervals. The resulting melting curve was used to determine the specificity of the PCR products. Some capillaries in the rotor were run in parallel for HumGAPD amplification, and the HumGAPD mRNA concentration in each sample was simultaneously determined to correct for differences in the amount and quality of total RNA. The relative amounts of HumBcl-2 and HumGAPD transcripts for all samples were calculated automatically using a standard curve. Amplification of the primers for HumBax was performed by the same procedure described above, except for elongation of the time at 72°C and the fluorescence acquisition temperature.

The sequences of the primers used for the rat genes were as follows: 5′-GAT GAC TTC TCT CGT CGC TA-3′ and 5′-GTC ATC CAC AGA GCG ATG TT-3′ for Rattus norvegicus B-cell leukemia/lymphoma 2 (Bcl2, termed RatBcl-2 in this study) from nucleotide 529 to 758 in the RatBcl2 sequence (accession no. NM-016993), 5′-CCG CTA ACA TCA AAT GGG GT-3′ and 5′-GGA TGC AGG GAT GAT GTT CT-3′ for Rattus norvegicus glyceraldehyde-3-phosphate dehydrogenase (GAPDH, termed RatGAPDH in this study) from nucleotide 1088–1473 in the RatGAPDH sequence (accession no. AF106860), and 5′-ACA GTT CAT GAA GAC AGG GG-3′ and 5′-CAA AGA TGG TCA CTG TCT GC-3′ for Rattus norvegicus bcl2-associated X protein (Bax,termed RatBax in this study) from nucleotide 51 to 529 (accession no. NM-017159).

Amplification of RatBcl-2, RatBax, and RatGAPDH was performed by the same procedure as for HumBcl-2 described, except for elongation of the time (20 seconds at 72°C) and a fluorescence acquisition temperature of 86°C.

2.6. Analysis of real-time RT-PCR data

A second derivative maximum method was used to determine the crossing points automatically for individual samples [29]. The fluorescence signal was plotted against the cycle number for all samples and the external copy number standards. For the external copy number standards, 2-fold serial dilutions of cDNA (corresponding to the starting copy numbers per 1 μg of total RNA) were used in each run. The relative amounts of target and reference genes were calculated based on crossing-point analysis (LightCycler analysis software version 3.39) of serial dilutions of the samples. To correct for differences in RNA quality and quantity between the samples, data were normalized using the ratio of the target cDNA concentration to that of GAPDH, which was assessed by a different reaction in the same experimental round.

2.7. Statistical analysis

All data were nonparametric, thus medians and interquartile ranges were used. A 2-tailed Mann-Whitney *U* test was used to compare significant differences between the caffeine-treated and non–caffeine-treated groups.

3. Results

The temporal changes of the transcription associated with the response of BeWo to 48 hours of caffeine treatment were screened on a cDNA microarray, and the balanced differential expression level in caffeine-nontreated controls *versus* caffeine-treated samples for HumBcl-2 was found to decrease [20]. HumBcl-2, one of the anti-apoptosis regulators, plays an important role in preventing apoptosis of trophoblasts in the human placenta. The microarray survey results suggested that caffeine down-regulates the mRNA expression of HumBcl-2 in human trophoblast-like cells. We selected the genes of HumBcl-2 and HumBax, another member of the bcl-2 family for further study of the effect of caffeine on responsive gene expression using a LightCycler PCR method and real-time detection system. In addition, we investigated *in vivo* mRNA expression of caffeine-induced placental bcl-2 genes.

3.1. Semiquantitative mRNA expressions of bcl-2 family-related genes in BeWo cells using real-time PCR system

A recently developed technique using the LightCycler system allows real-time detection of a specific PCR product by measuring fluorescence in the PCR exponential phase followed by post-PCR melting curve analysis. Continuous detection of the accumulating PCR products in each cycle allows for rapid and reliable quantification of gene expression. We used this real-time PCR system to estimate the mRNA expression level of the bcl-2 gene family using BeWo cells treated with 1.0 mmol/L of caffeine for 48 hours, and adapted the technology for a semi-quantitative RT-PCR assay using the double-strand DNA binding dye SYBR Green I, with the primers designated in the Methods and materials section. Furthermore, the specificity of the desired PCR products was confirmed by melting curve analysis. To quantify the HumBcl-2 transcript, we calculated the concentrations relative to mRNA encoding the HumGAPD housekeeping gene, allowing for normalization of target gene expression and correcting for differences in RNA quality and quantity between samples. As a result, we found a linear relationship between cycle number and logarithm of the determined concentrations of the standard HumGAPD cDNA, which showed a test linearity ranging from 1:10 to 1:160 (data not shown). The same linearity was also observed for the target HumBcl-2 cDNA (Fig. 1A).

To determine the precise concentration of the reference HumGAPD gene serial dilutions, which ranged from 1:10 to

1:160, each sample was analyzed automatically on the LightCycler and the results used to construct separate calibration curves. The same amount of quantification was also observed for HumBcl-2. Figure 1B shows typical analysis results, and Fig. 1C shows melting curve profiles of the PCR end-products of the HumBcl-2 cDNA template from BeWo cells. Amplification products showed a characteristic melting behavior for all concentration references (a–d), as well as for typical non–caffeine (*x*) and caffeine-treated (*y*) samples. The melting temperature (Tm) of the HumBcl-2 PCR product was 89.2°C, whereas the DNA artificially synthesized in the non-template control had a characteristically lower Tm, indicating amplification of smaller nonspecific DNA fragments (probably primer dimers) at later stages of the PCR run. However, these nonspecific products were not measured in the quantification step, as fluorescence acquisition was started at a higher temperature (>87°C).

We simultaneously amplified the HumGAPD template in each sample and obtained characteristic melting curves (data not shown). The HumGAPD PCR product showed only a single sharp peak at Tm = 88.5°C, which was clearly separated from the nonspecific product and primer-dimers.

HumBcl-2 mRNA was referred to as HumGAPD mRNA in each sample. The normalized HumBcl-2 value against HumGAPD is shown in Fig. 2 and Table 1. The HumBcl-2 mRNA expression BeWo cells treated with caffeine for 48 hours was significantly decreased from that of non–caffeine-treated control cells ($P < 0.0027$, Mann-Whitney *U* test).

To quantify the HumBax transcript, we performed the same protocol described above and obtained characteristic melting curve profiles of PCR end-products from the HumBax cDNA template from BeWo cells. The HumBax PCR product showed a single sharp peak at Tm = 88.1°C, which was clearly separated from the nonspecific product and primer dimers (Fig. 3). We referred the HumBax mRNA to the HumGAPD mRNA in each sample, and normalized the HumBax value to the HumGAPD; however, we found that caffeine had no effect on the expression of the HumBax gene (Table 2).

3.2. In vivo mRNA expressions of caffeine-induced placental genes in bcl-2 family

We expanded our study to investigate gene expressions in the placentas of pregnant rats that were fed a diet supplemented with caffeine for 20 days during gestation, and analyzed the results using the LightCycler system.

Melting curve profiles of the PCR end-products of the RatBcl-2 and RatGAPDH cDNA templates from the placentas were obtained. Both showed a characteristic melting behavior, which was expressed as a plot of the negative first derivative (−dF/dT) of the fluorescence *versus* temperature, and revealed the high purity and homogeneity of the PCR product (data not shown). The melting temperatures for RatBcl-2 and RatGAPDH were Tm = 83.3°C and Tm =

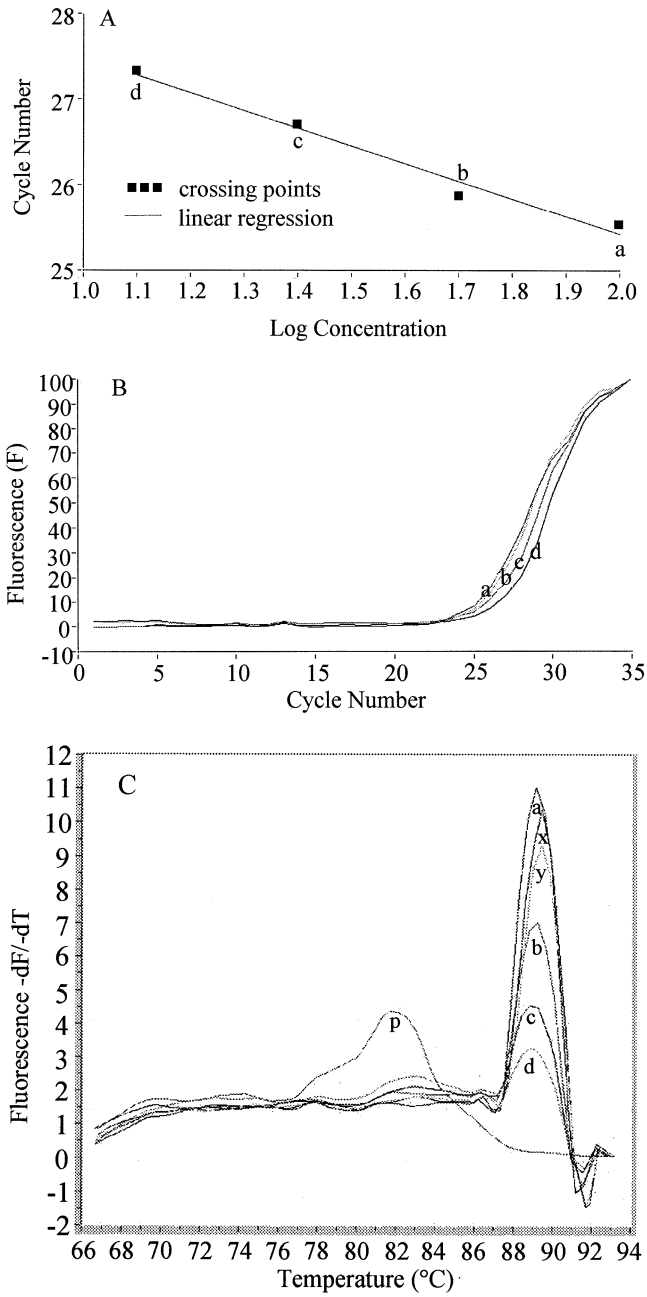

Fig. 1. Quantitative assay for HumBcl-2. A representative standard curve for HumBcl-2 mRNA from BeWo cells is shown (*A*). The initial dilutions of cDNA samples ranged from 1:10 (a) to 1:80 (d). The linear regression of the standard curve shown is based on the relationship between the logarithm of the template dilution and the cycle number of PCR crossing points. Amplification curves for serial dilutions of cDNA corresponding to (a) to (d) are indicated (*B*). The cycle number at which the tangent of exponential amplification crosses the fluorescence baseline at zero was defined as the PCR crossing point and reflects the relative amount of template in the reaction. A melting curve profile of RT-PCR end-products from HumBcl-2 is shown (*C*). The graph displays changes in SYBR Green I fluorescence *versus* temperature (−dF/dT) from the cDNAs of the copy number standards (a–d), as well as for typical caffeine nontreated (*x*) and caffeine-treated (*y*) BeWo cells simultaneously amplified for 38 cycles. PCR products from the non–template-negative control are indicated as p.

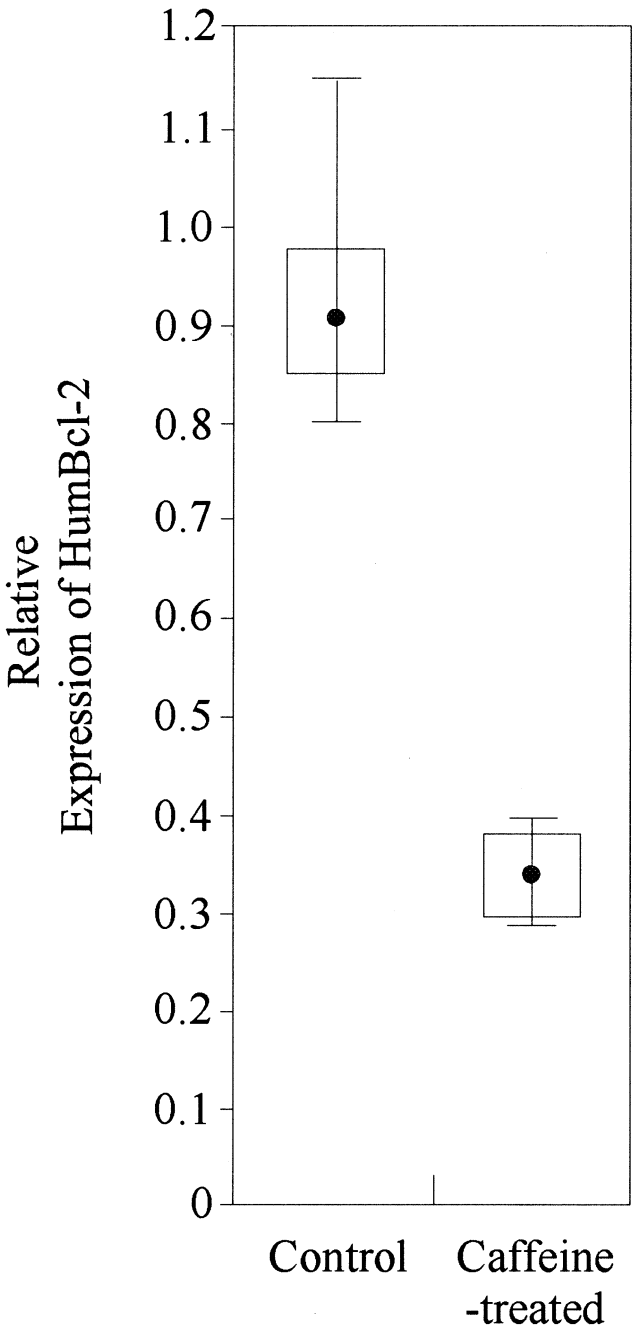

Fig. 2. Effect of caffeine on HumBcl-2 expression in BeWo cells. The normalized HumBcl-2 value against HumGAPD is shown. Results represent the medians (closed circle), and interquartile ranges within 25% and 75% (box), and within 10% and 90% (bars), of the relative expression of HumBcl-2 obtained in triplicate cultures (both control and caffeine-treated) from duplicate experiments. The HumBcl-2 mRNA expression of caffeine-treated BeWo cells was significantly different from that of non–caffeine-treated control cells ($P < 0.0027$, Mann-Whitney U test).

88.7°C, respectively. The synthesized DNA in the nontemplate control had a characteristically lower Tm (<76°C), indicating amplification of nonspecific smaller DNA fragments at later stages of the PCR run. In the fluorescence acquisition, each cycle was measured at 79°C in the fourth segment during the amplification program, and the fluorescence signal against the cycle number for the standards and samples was then plotted. RatBcl-2 and RatGAPDH transcripts introduced at the start of the reaction were calculated by plotting the cycle threshold of the unknown samples on the standard curve, as described above. The final result was the normalized RatBcl-2 value, which was expressed as the ratio between RatBcl-2 and RatGAPDH (Fig. 4 and Table 1). When the pregnant rats received caffeine during gestation, the expression of RatBcl-2 mRNA was significantly decreased from the level of noncaffeine administered control rats ($P < 0.00096$, Mann-Whitney U test).

In addition, mRNA expression of the RatBax cDNA template from the placentas was analyzed using the LightCycler system. We found that caffeine had no effect on expression of the RatBax gene (Table 2).

Table 1
Summary of Bcl-2 expression in caffeine treatment

Time of Caffeine Treatment	Gene Name	cDNA Microarray*	LightCycler System†
In vitro expression after 48-h caffeine treatment	HumBcl-2	2.54	2.78‡
In vivo expression after 20-day caffeine treatment during gestation	RatBcl-2	ND	2.06§

* Ratio of caffeine treated samples over caffeine-nontreated control levels.

† Ratio of caffeine treated samples over caffeine-nontreated control levels.

‡ Significantly different from the level of caffeine-nontreated cells at $P < 0.0027$.

§ Significantly different from the value of caffeine-non-administered control rats at $P < 0.00096$.

ND = not determined.

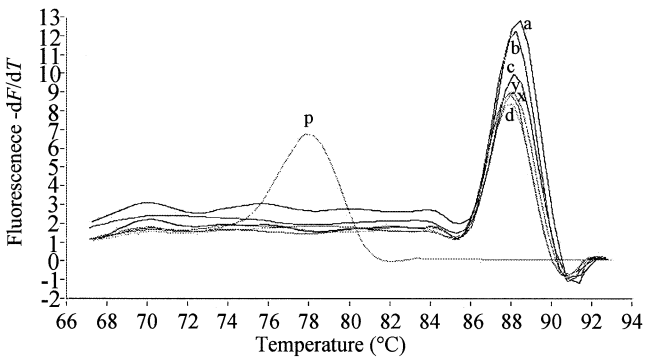

Fig. 3. Melting curve profiles of PCR end-products from the HumBax template. The initial dilutions of cDNA samples ranged from 1:10 (a) to 1:80 (d), as described in the legend to Fig. 1, and the PCR products from the non–template-negative control are indicated as p. cDNA of the standards as well as that of the non-treated (x) and caffeine-treated (y) BeWo cells were simultaneously amplified for 35 cycles. The graph shows changes in SYBR Green I fluorescence *versus* temperature (−dF/dT) for HumBax.

Table 2
Summary of Bax expression in caffeine treatment

Time of Caffeine Treatment	Gene Name	LightCycler*
In vitro expression after 48-hour caffeine treatment	HumBax	0.94
In vivo expression after 20-day caffeine treatment during gestation	RatBax	0.86

* Ratio of caffeine treated samples over caffeine-nontreated control levels.

4. Discussion

The present study demonstrated for the first time the effects of caffeine on mRNA expression by bcl-2 family genes, Bcl-2 and Bax, from the human cytotrophoblast-like cell line BeWo as well as in placentas from pregnant rats that were fed a caffeine-supplemented diet during gestation.

Bcl-2 is a membrane protein that suppresses apoptosis in response to a variety of stimuli [32], and has been found in some developmental stages of villous cytotrophoblasts [33–38].

To regulate the cell dynamics of placental villi, apoptosis plays a central role in villous trophoblast turnover and remodeling, and its cascade is initiated in villous cytotrophoblasts, which in turn promotes syncytial fusion, although its progression along this cascade is inhibited by Bcl-2 [36,39]. Increased apoptosis and decreased Bcl-2 expression have been reported in the placentas of pregnant women with IUGR [24,26,28,33]. Bax, an apoptosis promoter, is widely expressed *in vivo* and accelerates apoptotic cell death [40]. Furthermore, levels of the pro-apoptosis Bax protein can be post-traslationally regulated by Bcl-2, probably in a tissue-specific fashion, suggesting the existence of a feedback mechanism that may help to maintain the ratio of Bcl-2 to Bax protein in a physiologically appropriate range [41,42]. It has been postulated that Bcl-2 may be a type of proliferation or maturation-related marker of trophoblasts, which shows decreased expression along with terminal differentiation and maturation [33], and that susceptibility of a cell to undergo apoptosis is controlled through the interaction of Bcl-2 and Bax [40,43].

In the present study, we found a significantly decreased expression of Bcl-2 mRNA due to caffeine exposure during gestation in an animal model. Ishihara et al. [28] reported that IUGR is associated with an increase in placental apoptosis, and a decreased expression of Bcl-2 resulted in an increase of apoptosis in those specimens. We speculated that the decreased Bcl-2 expression found in the placentas removed from caffeine-treated pregnant rats in the present study might have represented a biological change from the further progression of apoptosis, resulting in IUGR. On the other hand, we did not find an alteration of Bax mRNA expression in either BeWo cells or rat placentas from caffeine-treated and nontreated samples.

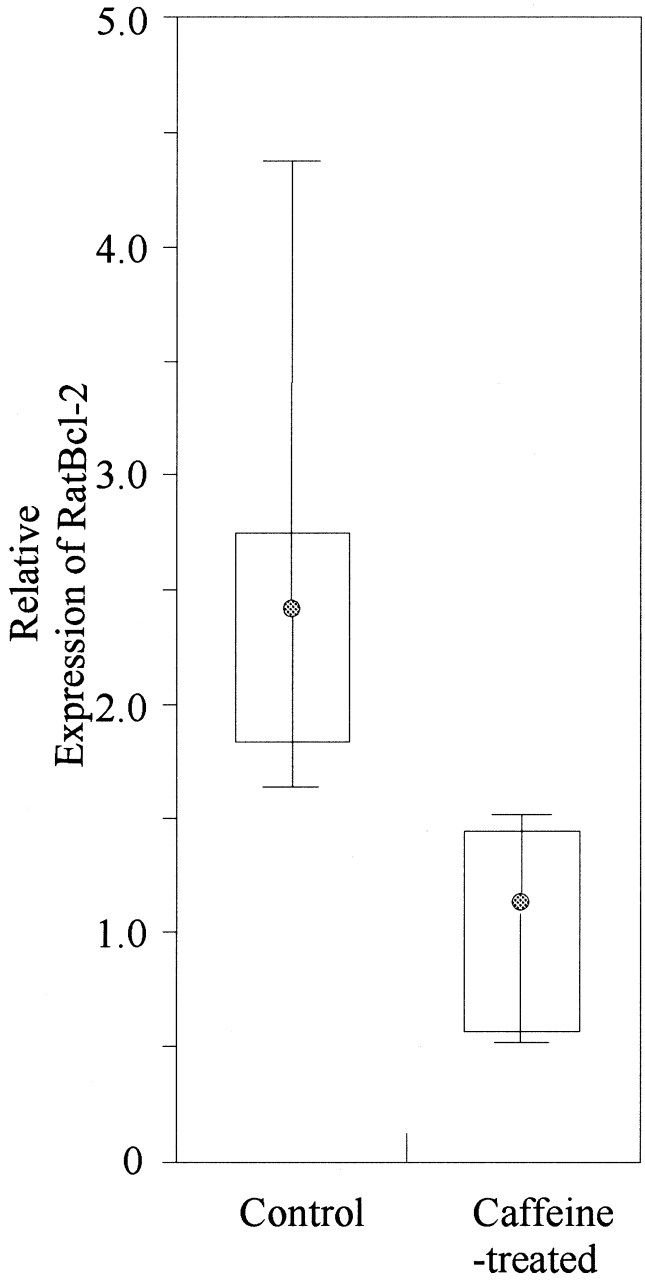

Fig. 4. Effect of caffeine on RatBcl-2 expression in the rat placentas. Normalized RatBcl-2 values against those of RatGAPDH are indicated. The results represent the medians (closed circle), as well as interquartile ranges within 25% and 75% (box), and within 10% and 90% (bars), of the relative expression of RatBcl-2 from three experiments performed with six placentas (from both control and caffeine-administered rats). The placental RatBcl-2 values of caffeine-treated rats were significantly different from the those of the nontreated controls ($P < 0.00096$, Mann-Whitney U test).

The amount of caffeine consumed by rats in the present study was equivalent to human intake of approximately 2 cups of coffee per day, as the half life of plasma caffeine in rats is much shorter than that in adult humans [44]. When the conversion is based on metabolic body weight [45], it

becomes slightly more than 2 cups per day. Many women of child-bearing age consume daily caffeine equivalent to that found in 2–4 cups of coffee [46,47]. Thus, the dosage used in the current *in vivo* studies was considered to be very conservative. In fact, the body weight of the fetuses in the present study showed no difference between groups.

It has not been shown whether caffeine consumption during pregnancy causes neonates to be smaller than their gestational age, as a recent study of caffeine intake reported no adverse effects on fetal growth [14], whereas another [48] found that fetuses were smaller than their gestational age in the heavy coffee drinker group, which was composed of subjects who consumed more than 5 cups of coffee each day. The present data demonstrated that a commonly consumed amount of caffeine intake during gestation altered gene expression. Had we used an amount of caffeine equal to that of heavy drinkers, the effects may have been much greater.

An investigation of the maternal ingestion of caffeine comparable to 2 cups of coffee during the last trimester found that intervillous placental blood flow significantly decreased [49]. The successful outcome of mammalian pregnancies is dependent upon an orchestrated development of both uteroplacental and fetoplacental circulations to meet the growing fetus oxygen and nutrients. The increase in uteroplacental blood flow at term gestation ensures that the delivery of oxygen and nutrients to the fetoplacental unit is adequate for normal fetal growth and development [50], therefore the impaired flow could lead to IUGR [51,52].

In a previous study of the role of caffeine on placental gene expression, we reported its effect on the placental renin-angiotensin system, by significantly enhancing AT_2 receptor gene expression, and suggested that increased AT_2 receptor expression during gestation might impair the balance of the vasoconstrictor and vasodilator in the system that maintains the blood flow in the placental bed, resulting in the LBW babies [20,21]. It has also been reported that the up-regulation of AT_2 receptor results in the inactivation of Bcl-2 and induction of apoptosis [49]. The present findings demonstrated that caffeine caused a decreased level of Bcl-2 expression in a human trophoblast cell line and placentas removed from caffeine-administered pregnant rats.

The precise mechanisms of how caffeine interacts with the AT_2 receptor and the Bcl-2 to induce the alteration of these gene expressions on the cell surface of the placental cytotrophoblast are not clear. However, one could put forth that caffeine may enhance AT_2 receptor gene expression to impair the uteroplacental blood flow, and induce a further progression of placental apoptosis via inactivation of Bcl-2 gene expression, resulting in IUGR.

In addition to our findings, the effects of caffeine consumption by drinking beverages such as tea and coffee during pregnancy have been reported [6,7,10]. Many unknown adverse developments in various organs could result in later life as a result of chronic caffeine exposure by the fetus, possibly through the activation of the AT_2 receptor and inhibition of Bcl-2 gene expression during the critical time of growth [53–55]. Therefore, further studies of the effect of caffeine during pregnancy are required to fully elucidate this potential health problem.

Acknowledgments

We thank Dr. Hiroyuki Sakamaki for his technical support and helpful discussion. This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology to promote 2001-multidisciplinary research projects (in 2001-2005), B2-13470395, and by the Nutritional Division of Ross Laboratories in the USA.

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