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Benzo(a)Pyrene Causes PRKAA1/2-Dependent ID2 Loss in Trophoblast Stem Cells

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SUMMARY

Benzo(a)pyrene (BaP), a cigarette smoke component, is metabolized to diol esters (BPDE) that bind to DNA and form mutagenic BPDE-DNA adducts. BaP activates stress enzymes including stress-activated protein kinase/jun kinase (MAPK8/9) in embryos, AMP-activated protein kinase alpha1/2 subunits (PRKAA1/2) in somatic cells, and inhibits the proliferation of trophoblast cell lineages. The loss of transcription factor inhibitor of differentiation (ID)2 is required for the initial differentiation of mouse trophoblast stem cells (TSC) in implanting mouse embryo to produce the first placental hormone, chorionic sommatomammotropin (CSH)1. Here we demonstrate that BaP activates PRKAA1/2 and causes ID2 protein loss in TSC in a time- and dose-dependent manner. Although PRKAA1/2 was activated at low BaP doses, PRKAA1/2-dependent ID2 protein loss occurred at a dose that was similar to the threshold that results in a significant decrease in TSC accumulation and decreased fraction of proliferating TSC. This suggests a possible relationship between stress-induced declines in cell accumulation and stem cell differentiation when BaP levels are high. The threshold BaP dose that induces significant ID2 loss is in the range of a 2–3 pack/day habit, suggesting that this mechanism may be involved with implantation failure in smoking women.

INTRODUCTION

It has been demonstrated that cigarette smoke components are pathogenic for fertility and pregnancy. These adverse effects include decreased success rate of in vitro fertilization,

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spontaneous abortion, intrauterine growth restriction, and low birth weight (Higgins, 2002; Zdravkovic et al., 2005; Rappolee et al., 2010).

There are thousands of components in cigarette smoke, and benzo(a)pyrene (BaP) is an important, mutagenic, and toxic one (Witschi, 2001). BaP is metabolized to diol esters (BPDE) that bind to DNA and form the BPDE-DNA adducts that disrupt DNA replication and cause mutations. Several in vivo studies have shown that BPDE-DNA adducts accumulate on the placenta or cross the placenta barrier, producing toxicity to fetal and placental development (Everson et al., 1988; Arnould et al., 1997; Sanyal et al., 2007). In vitro studies have shown effects of BaP on the proliferation of trophoblast cell lineages. In those investigations, BaP significantly inhibited proliferation of human trophoblast cell lines by suppressing the cell cycle and activating G2/M cell cycle checkpoints, myelocytomatosis oncogene (MYC), epidermal growth factor receptor (EGFR), and activation of transformation related protein (TRP)53, $p21^{cip1}$, transforming growth factor (TGF)β1 (Zhang et al., 1995; Zhang and Shiverick, 1997; Drukteinis et al., 2005; Rappolee et al., 2010).

The trophoblast lineage first arises in preimplantation embryos, the source of pluripotent placental trophoblast stem cells (TSC) (Chai et al., 1998; Tanaka et al., 1998). In recent years, the genetic program for mediating TSC differentiation to giant cells has been extensively investigated. In normal TSC differentiation, the dominant negative inhibitor of differentiation (ID)2 is lost, allowing heart and neural crest derivatives (Hand1) to bind transcription factor 3 (TCF3, also known as E12 or E47) and the resultant heterodimer activates the chorionic sommatomammotropin [CSH1, also known as placental lactogen (PL)1] promoter (Cross et al., 2002). Similarly in humans cytotrophoblast differentiation to the invasive state soon after implantation requires the loss of ID2 (Janatpour et al., 2000), although later differentiation events may require ID2 (Liu et al., 2004). ID2 is present during preimplantation development (Cross et al., 1995), and presumably loss of ID2 and gain of Hand1 drives trophoblast differentiation in the mouse. We test here if stress triggered by BaP affects ID2.

Individual cigarette smoke components affect periimplantation embryo development. The primary cigarette tar benzo-(a)pyrene (BaP) blocks implantation of embryos from normal mice with an EC50 of 0.33 μM (Iannaccone et al., 1984). In addition, the preimplantation embryo can metabolize BaP to its mutagenic diol ester (BPDE) (Filler and Lew, 1981, reviewed in Rappolee et al., 2010). Thus the preimplantation embryo and its TSC are susceptible to direct or indirect pathogenesis due to BaP.

Protein kinase AMPK-activated alpha subunit 1/2 (PRKAA1/2, also known as AMPKa1/2) and PRKAA1/2 gene family members are expressed and active in oocytes and during early embryonic cleavage divisions in mice (Heyer et al., 1997; Wang et al., 2004; Chen and Downs, 2008). PRKAA1/2 is also induced by hyperosmolar sorbitol-mediated cellular stress in oocytes (LaRosa and Downs, 2006) and leads to oocyte maturation. BaP activates stress enzymes such as stress-activated protein kinase/jun kinase (MAPK8/9, also known as stress-activated protein kinase/jun kinase, SAPK/JNK), p38 mitogen activated protein kinase (MAPK14, also known as p38MAPK), PRKAA1/2 in adult somatic cells (Patten Hitt et al., 2002; Li et al., 2004; Du et al., 2006; Mizrachy-Schwartz et al., 2007) and MAPK8/9 in embryos (Xie et al., 2008). Thus it is likely that stress enzymes such as PRKAA1/2 and MAPK8/9 mediate homeostatic and developmental responses to a wide range of pathogenic stimuli, and are important in mediating the stress response in embryos and TSC induced by BaP.

It is now widely accepted that BaP inhibits the proliferation of trophoblast cell lineages (Rappolee et al., 2010). However, very few studies have been done to address the effects of cigarette smoke components on the TSC differentiation (Genbacev et al., 1995). Because maternal smoking may fundamentally alter placental development and cause pregnancy complications by influencing the differentiation of TSC, it is important to determine the effects of BaP on TSC differentiation. Therefore, we tested if BaP activates PRKAA1/2 and regulates ID2, the transcription factor whose loss is required for the initial differentiation of mouse TSC to produce the first placental hormone after implantation, CSH1.

RESULTS

We first tested for the biological effects of benzopyrene on TSC accumulation at doses near the EC50 (0.33 μM) that negatively impact embryo implantation (Iannaccone et al., 1984) and at doses known to activate stress enzymes such as MAPK8/9 in preimplantation embryos (1 μM) (Xie et al., 2008). TSC exposed to all BaP concentrations for 24 hr had significantly more cells than at time zero (ANOVA, $P < 0.05$) (Fig. 1A). Although, BaP at 0.25 μM had no significant effect on cell accumulation of TSC (post hoc Dunnett *t*-test, $P=0.6$), 2.5 μM BaP significantly decreased cell accumulation (post hoc Dunnett *t*-test, $P < 0.001$), and 1.0 μM BaP causes nearly significant cell accumulation (post hoc Dunnett *t*-test, $P = 0.07$). Thus, TSC continued to accumulate at all BaP doses, and significantly diminished TSC accumulation at high doses.

The lowest BaP dose that significantly reduced BrdU-positive cells and increased cleaved caspase 3 (CASP3) was 0.5 μM. (one way ANOVA followed by Student-Newman–Keuls post hoc test) (Fig. 1B,C). Thus, markers for S phase commitment in embryos and TSC (Xie et al., 2006a; Zhong et al., 2007) and apoptosis in embryos and trophoblast cells (Dash et al., 2003; Mullen and Critser, 2004) have a dose response range that is similar to cell accumulation. The PRKAA1/2 metabolic substrate acetyl CoA carboxylase (ACACA, also known as ACC) was also phosphorylated and activated at a threshold dose of 0.5 μM BaP (Fig. 1D) in a PRKAA1/2-dependent manner (data not shown).

We next tested for the time-dependence of PRKAA1/2 induction by BaP. PRKAA1/2 phosphorylation at Thr172 increased significantly from 10 to 120 min (post hoc Dunnett *t*-test, $P < 0.001$) with a peak at 2.5-fold over background at 30 min, subsided to background levels by 4 hr and remained at background through 24 hr (Fig. 2). PRKAA1/2 Thr172 reached a significant (post hoc Student-Newman–Keuls, $P < 0.005$) plateau stimulation of 2.0-fold over background at 0.25 μM that was consistent through 2.5 μM doses of BaP (Fig. 3). At 0.15 μM BaP, however, there was a significant increase above background (Supplemental Fig. 1) that was also significantly less than the plateau at 0.25 and 0.5 μM established in Figure 2. Note that PRKAA1/2 was detected as a single band at the correct size of 63 kDa when protein was size fractionated with a 4–25% gradient polyacrylamide gel electrophoresis (PAGE) gel, but a second lower band was also detected (Supplemental Figure 1) when proteins were size fractionated on a 10% PAGE gel.

Hyperosmolar sorbitol causes PRKAA1/2 Thr172 induction and ID2 loss in TSC (data not shown; Rappolee et al., 2010; Zhong et al., manuscript in preparation), so we next tested if ID2 loss occurred in BaP-stimulated TSC. BaP caused a significant loss of ID2 that started at 60 min and peaked at an 80% loss at 4 hr (post hoc Dunnett *t*-test, $P < 0.001$), but returned to baseline by 24 hr (Fig. 4). BaP caused ID2 loss in TSC by 2 hr, but loss did not occur at BaP doses through 0.25 μM, but ID2 loss became significant only at 1.0 μM to 2.5 μM BaP (Fig. 5, post hoc Student-Newman–Keuls test, $P < 0.005$).

ID2 maintains TSC potency and suppresses differentiation and hyperosmolar stress causes loss of ID2 in a PRKAA1/2-dependent manner (Zhong and Xie, manuscript in preparation). BaP at 1 μM induces significant loss of ID2 protein at 4 hr that was significantly reversed by 5 μM of compound C, suggesting a PRKAA1/2-dependent process (post hoc Duncan test, $P < 0.05$) (Fig. 6). For TSC cultured in media alone, the presence of the PRKAA1/2 inhibitor Compound C for a 2 hr preload and for the 4 hr of the experiment had no significant effect on ID2 protein quantity, and 1 μM BaP treated TSC with compound C were not significantly different than unstressed TSC.

It should be noted that there was variation between triplicate experiments for the ratio of PRKAA1/2 Thr172 and ID2 normalized to loading control. For example ID2 loss varied from 60% to 80% in Figures 4-6 and PRKAA1/2 Thr172 induction varied from 1.5 to 2.5 (and 1.5 to 2.0 for a single dose and duration). This could be due to several reasons, including changes in lots of serum, use of different lots of conditioned medium to culture TSC, or different confluences of TSC at the start of experimental stress. Differences in effects have been observed previously based on the density of TSC at the start of stimulation (Hemberger et al., 2004). Nonetheless, all variations were in quantitative degree and do not affect the conclusions that the data support.

DISCUSSION

BaP is Stressful in Activating PRKAA1/2 and Decreasing Expression of ID2

We show here that BaP activates PRKAA1/2 Thr172 and causes loss of ID2 protein in a PRKAA1/2-dependent manner. In mouse and humans, the loss of ID2 occurs normally and is essential for aspects of early postimplantation TSC differentiation. Thus, BaP could induce the preparation of TSC differentiation that mimics some of normal development. If BaP stress was sufficiently strong (in magnitude and duration), however, then it is possible that BaP would cause pathologic reduction of the pool of undifferentiated pluripotent TSC. This might produce either insufficient TSC for essential early endocrine function or produce sufficient endocrine activity but sacrifice later placental function in doing so.

The highest dose of BaP at 2.5 μM caused significantly decreased cell accumulation compared with TSC in media alone. BaP at 1 μM was nearly significant in causing decreased cell accumulation. Although PRKAA1/2 Thr172 is activated by concentrations of BaP much lower than 1 μM, significant loss of ID2 only occurs at ≥1 μM. Increased apoptosis, denoted by cleaved CASP3, and decreased cell cycle entrance, denoted by the S phase marker BrdU, occurred at doses of ≥0.5 μM BaP. Thus, significant PRKAA1/2-dependent loss of ID2 occurs at nearly the same BaP dose that results in a significant decrease in cell accumulation through increased apoptosis and decreased proliferation.

Although BaP caused a two-fold increase in PRKAA1/2 Thr172 from 0.25 to 2.5 μM, the peak dose was 0.25 μM. Further, there was a significant PRKAA1/2 Thr172 increase at 0.15 μM BaP that was less than that at 0.25 μM. The PRKAA1/2-dependent substrate ACC ser79 was also not phosphorylated until 0.5 μM BaP. Thus, PRKAA1/2 is activated at lower doses of BaP when ID2 is not lost and in this case ACACA was not phosphorylated.

Interestingly, for hyperosmolar cellular stress ACACA is phosphorylated at doses as low as the lowest significant activation of PRKAA1/2 (manuscript in preparation). Thus, for hyperosmolar stress there is a low dose range of PRKAA1/2 and PRKAA1/2-dependent ACACA phosphorylation and a high dose range of ID2 loss and cell accumulation decrease. This sort of metabolic adaptation to BaP stress may occur but does not include ACACA function.

Many studies have focused on the effects of cigarette smoking components on the proliferation of trophoblast lineages and have demonstrated that BaP and nicotine adversely affect trophoblast cell proliferation (Zhang et al., 1995; Zhang and Shiverick, 1997; Genbacev et al., 2000; Drukteinis et al., 2005, reviewed in Rappolee et al., 2010). Only a few studies have addressed the relationship between cigarette smoke components and trophoblast stem cell differentiation. Genbacev et al. reported that nicotine inhibited normal first trimester cytotrophoblasts invasion, apparently by reducing the ability of treated cells to synthesize and activate type IV collagenase (Genbacev et al., 1995). The study here associates decreased TSC proliferation with loss of ID2, the latter being an event that normally occurs prior to TSC differentiation and CSH1 secretion.

Our data showed that BaP caused loss of ID2, indicating BaP may favor TSC differentiation. Our data raise questions for future investigations: Will this loss of ID2 lead to gain of Hand1 function, induction of CSH1, and eventually to TSC differentiation? If so, is this BaP-induced TSC differentiation the same as differentiation in vivo, or differentiation induced by FGF4 removal in culture?

We also studied the kinetic response of TSC to BaP. There was a rapid, 2.5-fold increase in PRKAA1/2 Thr172 by 30 min in 1 μM BaP. The PRKAA1/2 response peaked at 10–30 min and returned to baseline by 4 hr of stimulation. At 24 hr PRKAA1/2 Thr172 remained at the baseline, unstressed level. This indicates that despite the presence of BaP, TSC did not continue to activate PRKAA1/2 after 4 hr. Thus, the data suggest that the TSC have adapted to BaP, and PRKAA1/2 activity may be not necessary for the continuing response to BaP.

There are caveats to the pharmacokinetic response of a single cell type cultured in monolayer compared with BaP effects on multiple cells types in vivo. In cultured TSC, the loss of PRKAA1/2 from 4 to 24 hr and the accumulation of ID2 from 4 to 24 hr after the initial PRKAA1/2-dependent loss suggests a direct, reciprocal relationship between ID2 levels and PRKAA1/2 activity. In other studies we have shown that ID2 mRNA remains at 100% of levels in unstressed TSC during 24 hr of hyperosmolar stress (Liu et al., 2009). Hyperosmolar stress causes PRKAA1/2-dependent ID2 protein loss mediated by proteasomes (Zhong et al., manuscript in preparation). If BaP and hyperosmolar stress are similar in PRKAA1/2-dependent ID2 loss, then the return of ID2 protein after 24 hr of BaP may derive from ID2 mRNA that remains during the stress.

Relevance of BaP Levels Tested in Cultured TSC Compared With BaP Metabolite Levels in Smoking Women

These studies were performed to understand if cigarette smoke components regulate stress enzymes and transcription factors that regulate differentiation in peri- implantation TSC. It is important to understand the pathogenic doses of BaP in vivo and compare these to the dose-dependent nature of TSC differentiation observed here. BaP levels were tested by gas chromatography in ovarian follicular fluid of women in IVF therapy exposed to mainstream smoke and found to average 1.8 ng/mL (Neal et al., 2007). For women who smoked 12–24 cigarettes/day, BaP was 4–10 ng/mL in follicular fluid or twice as high in serum. However, consideration of BaP metabolites increased this range by as much as 10-fold (40–100 ng/mL). At 252 gm/M for BaP, 100 ng/mL is about 0.4 μM. This is a concentration that activated PRKAA1/2 in our study, but is subthreshold for ID2 loss and decrease in cell accumulation. Thus, typical cigarette habits in the range of 1/2–1 pack/day would cause a homeostatic effect, but not a developmental one. However it is possible that a 2–3 pack a day habit would elevate cellular stress to levels that would decrease TSC accumulation and ID2 levels. Of course other cigarette smoke components may mollify or exacerbate the effects of BaP.

We conclude that cigarette smoke may increase the risk of pregnancy complications by fundamentally affecting TSC potency and differentiation. Stress enzyme activation is likely to play an important role in mediating the contribution of BaP to stress-induced TSC differentiation. The next phase of research should investigate if BaP leads to induction and maintenance of Hand1 with a resultant induction of PL1 as occurs with hyperosmolar stress (Zhong et al., manuscript in preparation). Investigation of the functions of TSC after BaP-induction is also very important. Regulation of cell accumulation and the developmental substrate ID2 occurs in a PRKAA1/2-dependent manner at high BaP doses, but what is the function of PRKAA1/2 at lower BaP doses? At lower doses of hyperosmolar stress PRKAA1/2 phosphorylates its homeostatic substrate pACACA, (Zhong et al., manuscript in preparation). This is different than BaP stimulation, where significant ACC phosphorylation occurs at higher doses. Finally, the mouse TSC models some events in the implanting embryo, so it will be very important to test for the in vivo effects of BaP and cigarette smoke on the stress and stress enzyme functions in regulating growth and potency of stem cells in the implanting embryo.

MATERIALS AND METHODS

Reagents

FGF4, heparin, and benzo(a)pyrene were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum and RPMI1640 were from Gibco (Grand Island, NY). The primary antibodies for total ID2 (SC489) and (ZMD325) were from Santa Cruz Biotechnology (Santa Cruz, CA) and Zymed (South San Francisco, CA), respectively. Antibodies for PRKAA1/2 Thr172 (CS2531, CS2535) and (SC33524R), and PRKAA1/2 substrate ACACA Ser79 (CS3661) were from Cell signaling (Danvers, MA) and Santa Cruz, respectively. Actin antibodies (CS4967) and cleaved CASP3 (CS9664) were from Cell signaling. The specific inhibitor for AMPK, Compound C (Zhou et al., 2001) was purchased from Calbiochem (San Diego, CA). Bromodeoxyuridine (brdU) was purchased from BD Biosciences/Pharmingen (San Diego, CA) and monoclonal antibodies were purchased from Santa Cruz (SC32323). The brdU monomers were purchased from BD Pharmingen (San Diego, CA). Control PRKAA1/2 lysates (CS9158) from hydrogen peroxide stimulated C2C12 cells were purchased from Cell signaling.

Cell Lines and Culture Conditions

The mouse TSC cells were from Dr. Rossant (Lunenfeld Research Institute, Ontario, Canada) and cultured as described previously (Xie et al., 2005). Cells were cultured with benzo(a)pyrene diluted from a 2 mg/mL stock (2,000-fold concentrate) and used at a final concentration of 1 μM in media.

Cell Accumulation, Proliferation, and Apoptosis Assays

Cell accumulation was assayed by counting cells using a hemocytometer and fraction of cells in cell cycle was determined by BrdU incorporation as we have previously reported (Xie et al., 2006a, 2007; Zhong et al., 2007). Monoclonal antibodies to Bromodeoxyuridine were purchased from (catalog number M0744, Dako, Carpinteria, CA). CASP3 is commonly used to detect apoptosis in preimplantation embryos and trophoblast cells (Dash et al., 2003; Mullen and Critser, 2004).

Western Blot

Sodium dodecyl sulfate-polyacrylamide gel elecrophoresis (SDS-PAGE) and Western immunoblot analysis of blastocysts were performed as previously described (Wang et al., 2005; Xie et al., 2005, 2006b, 2007).

Statistical Analysis

The data in this study are representative of three independent experiments and indicated as mean±SD. The statistical significance of differences between treated samples was analyzed by One-way analysis of variance (ANOVA) for continuous data with more than two groups (SPSS 11.0). Dunnett *t*-test, Duncan, and Student-Newman–Keuls post hoc tests were used to analyze significance of pairwise comparisons. Groups were considered to be significantly different if $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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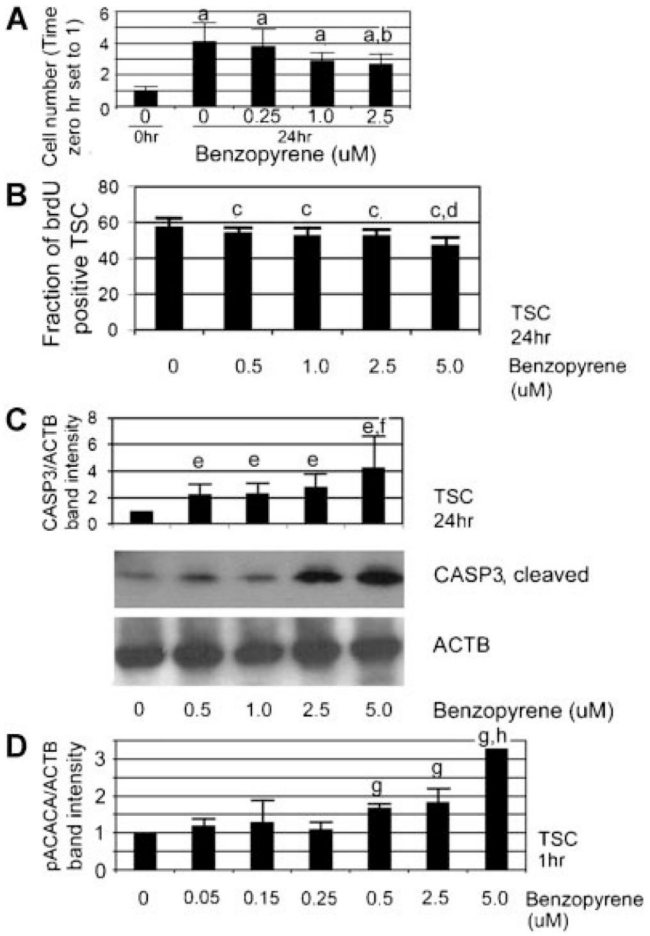

Figure 1.

BaP at 0.25 μM has no significant effect on TSC accumulation, but at 1 and 2.5 μM there is a significant decrease in TSC accumulation. TSC were cultured for 24 hr without BaP or with 0.25, 1, 2.5, or 5.0 μM BaP (**A**,**B**) and assayed for cell number (accumulation) or BrdU to quantify proliferating cells (Materials and Methods Section). The histogram shows the mean and SEM for cell counts of TSC at time zero and at 24 hr for the doses of BaP indicated (A) and for fraction of cells that are BrdU positive (B). Time zero was set to one. Error bars show the SEM of three experiments. **a**: Overall ANOVA shows significantly more cells at 24 hr for all doses taken together, than at 0 hr (a). **b**: Post hoc Dunnett *t*-test shows cell number for TSC cultured with 1 and 2.5 μM BaP for 24 hr is significantly lower than 0 μM at 24 hr. SNK post hoc tests show that (**c**) there were significantly fewer BrdU positive cells at 0.5, 1, and 2.5 μM BaP than in unstressed cells, and (**d**) shows there were significantly fewer BrdU positive cells at 5.0 μM than at lower BaP doses. TSC were stimulated with 0–5 μM BaP for 24 hr (**C**) or for 1 hr (**D**). Total protein from lysed cells were fractionated by SDS-PAGE, and immunoblotted for cleaved CASP3 (C) or ACACA Ser79 (D) and for actin (ActB, not shown for D). Histograms show triplicate experiments and error bars show SEM. **e**: For cleaved CASP3 SNK post hoc tests show that 0.5–2.5 μM causes significantly more apoptosis than no BAP and (**f**) 5 μM BaP causes more apoptosis than lower doses. **g**: For ACACA Ser79, 0.5–5 μM causes significant activation compared with unstressed TSC and (**h**) 5 μM BaP activates more ACACA Ser79 than lower doses.

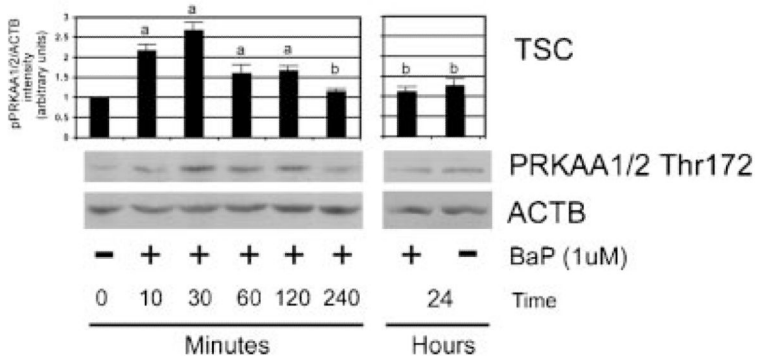

Figure 2.

BaP induces an early peak of PRKAA1/2 Thr172 phosphorylation at 30–60 min that subsides by 120 min and remains at baseline at 24 hr. TSC were cultured in 1 μM BaP for 0–24 hr, lysed, fractionated by SDS-PAGE and immunoblotted for PRKAA1/2 Thr172 or for ACTB as a loading control. In the histogram, error bars show the standard deviation for three experiments. Overall ANOVA shows significant PRKAA1/2 Thr172 induction for all BaP durations between 10 and 240 min taken together compared with unstressed TSC at 0 min. PRKAA1/2 Thr172 is induced significantly at 10–120 min using the post-hoc Dunnett *t*-test (**a**) ($P < 0.001$), but that PRKAA1/2 Thr172 is not significantly different in BaP-treated group at 4 hr (b). Post hoc Dunnett *t*-test shows no difference for PRKAA1/2 Thr172 when TSC are stimulated or unstimulated by BaP for 24 hr (b) compared with each other or with unstressed TSC at 0 min.

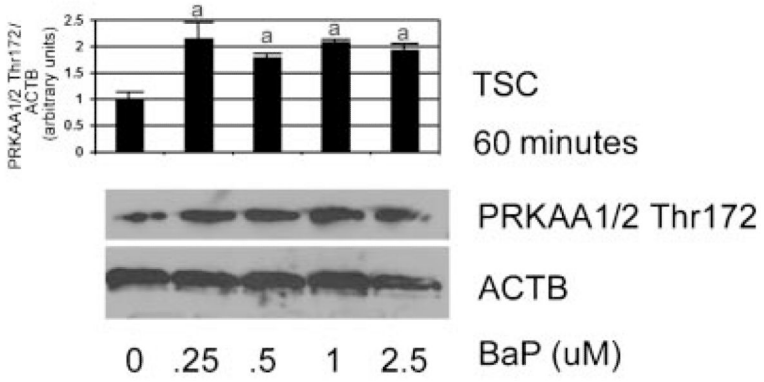

Figure 3.

BaP induces a two-fold increase in PRKAA1/2 Thr172 phosphorylation at 0.25 μM BaP that continues through 2.5 μM BaP. TSC were cultured in 0–2.5 μM BaP for 60 min, lysed, fractionated by SDS-PAGE and immunoblotted for PRKAA1/2 Thr172 or for ACTB as a loading control. In the histogram, error bars show the standard deviation for three experiments. Overall ANOVA shows significant PRKAA1/2 Thr172 induction for comparisons of unstressed TSC with all doses taken together at 1 hr. Post hoc Student-Newman–Keuls test also shows that each individual dose from 0.25 to 2.5 μM causes significant induction of PRKAA1/2 Thr172 at 1 hr ($P < 0.005$) compared with unstressed TSC (**a**).

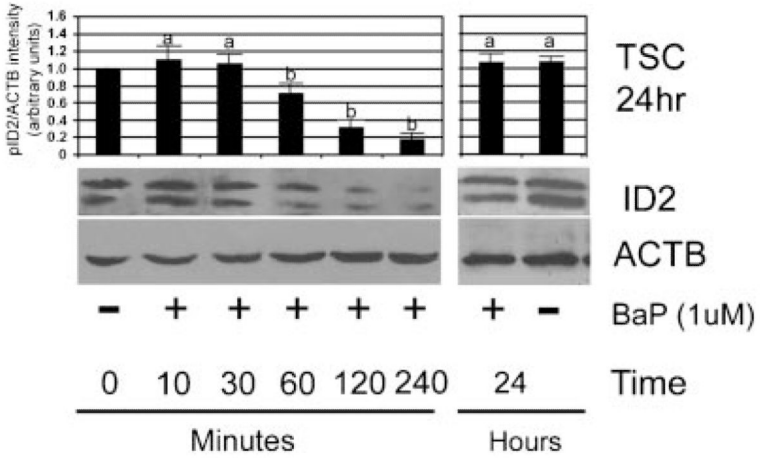

Figure 4.

BaP induces an early 80% loss of ID2 by 120 min that reverses at 24 hr. TSC were cultured in 1 μM BaP for 0–24 hr, lysed, fractionated by SDS-PAGE and immunoblotted for ID2 or for ACTB as a loading control. In the histogram, error bars show the standard deviation for three experiments. Overall ANOVA shows significant ID2 loss for all doses BaP durations taken together compared with unstressed TSC at 0 min, but post hoc Dunnett *t*-test shows that ID2 is significantly decreased at 60, 120, and 240 min ($P < 0.001$) (**b**), whereas 10 and 30 min are not significantly different than 0 min (**a**), and ID2 for BaP treated and untreated at 24 hr are not significantly different (a).

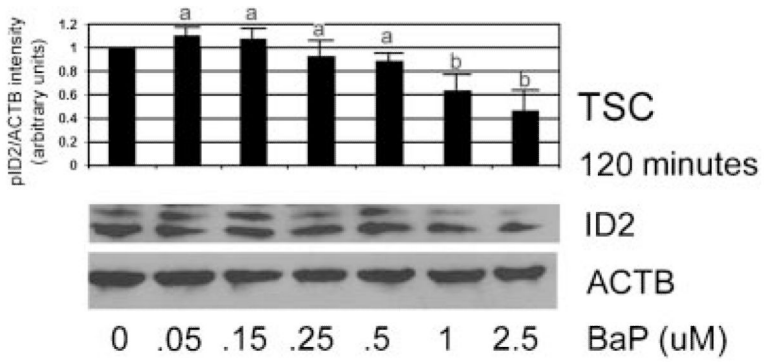

Figure 5.

BaP induces a dose-dependent ID2 loss that is maximized at 60% at 2.5 μM. TSC were cultured in 0–2.5 μM BaP for 2 hr, lysed, fractionated by SDS-PAGE and immunoblotted for ID2 or for ACTB as a loading control. In the histogram, error bars show the standard deviation for four experiments. Overall ANOVA shows significance of ID2 loss for comparisons of unstressed with all doses taken together at 2 hr ($P < 0.05$). Post hoc Student-Newman–Keuls tests show that 1 μM and 2.5 μM BaP cause significant loss of ID2 at 24 hr (**b**) ($P < 0.005$) but that 0.05–0.5 μM does not cause significant loss (**a**).

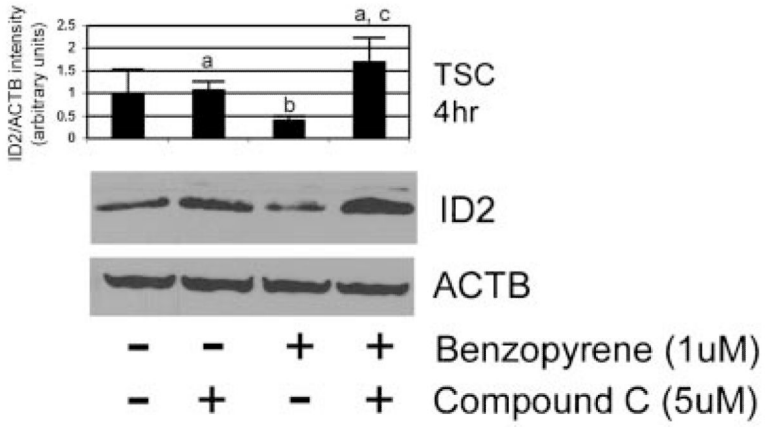

Figure 6.

BaP-induce loss of ID2 in a PRKAA1/2-dependent manner. TSC were cultured with or without 1 μM BaP and with or without 5 μM compound C for 4 hr, lysed, fractionated by SDS-PAGE and immunoblotted for ID2 or for ACTB as a loading control. In the histogram, error bars show the standard deviation of three independent biological experiments. Overall ANOVA shows nonsignificance at 4 hr for all comparisons taken together, versus media alone. But post hoc Duncan tests show that ID2 protein levels after 1 μM BaP are significantly lower than media alone (**b**) and that BaP stimulation during Compound C inhibition is significantly higher than BaP alone (both at $P < 0.05$) (**c**) whereas BaP + compound C, or compound C alone, is not significantly different than media alone (**a**).