Are Developing β-Adrenoceptors Able to Desensitize?

Acute and Chronic Effects of β-Agonists in Neonatal Heart and Liver

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Abbreviations: AC, adenylyl cyclase
ANOVA, analysis of variance
β-AR, beta-adrenoceptor
cAMP, cyclic 3’,5’-adenosine monophosphate
PN, postnatal day

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ABSTRACT

During fetal and neonatal development, β-adrenergic receptors (β-ARs) appear to be resistant to desensitization by β-agonist drugs. To determine the mechanisms underlying the regulatory differences between adults and neonates, we administered isoproterenol, a mixed β₁/β₂-AR agonist, and terbutaline, a β₂-selective agonist. Effects were examined in the ensuing four hours after a single injection, or after the last of four daily injections. We prepared cell membranes from heart (predominantly β₁-ARs) and liver (predominantly β₂-ARs), and assessed signal transduction in the adenylyl cyclase (AC) pathway. In the first few hours after a single administration of isoproterenol to adult rats, cardiac β-ARs showed activation of G-proteins (elevated AC response to forskolin) and desensitization of β-AR-mediated responses; after the fourth injection, heterologous desensitization emerged, characterized by a loss of signaling mediated either through β-ARs or glucagon receptors. Terbutaline evoked an increase in the forskolin response but no desensitization of receptor-mediated responses. When we gave the same treatments to neonatal rats, we observed cardiac G-protein activation, but there was neither homologous nor heterologous desensitization of β-ARs or glucagon receptors. In the adult liver, isoproterenol and terbutaline both failed to evoke desensitization, regardless of whether the drugs were given once or for four days. In neonates, however, acute or chronic treatment elicited homologous desensitization of β-AR-mediated AC signaling, while sensitizing the response to glucagon. These results show that neonatal β-ARs are inherently capable of desensitization in some, but not all cell types; cellular responses can be maintained through heterologous sensitization of signaling proteins downstream from the receptor. Differences from adult patterns of response are highly tissue-selective and are likely to depend on ontogenetic differences in subtypes of β-ARs and AC.
INTRODUCTION

Homeostasis requires the suppression of cellular responsiveness in the face of prolonged or excessive stimulation. For the β-adrenoceptor/adenyl cyclase (β-AR/AC) signaling cascade, cAMP levels plateau or return to basal levels during maintained agonist exposure through two mechanisms (30, 32, 49): uncoupling of β-ARs from their response elements (desensitization) and reductions in the concentration of receptors at the cell membrane (downregulation). With homologous desensitization, effects are restricted to β-AR signaling, typically through receptor phosphorylation by G-protein-coupled receptor kinases; phosphorylation blocks the coupling of β-ARs to G-proteins, and enables the binding of β-arrestin, leading to receptor internalization and consequent downregulation (17, 29). With heterologous desensitization, β-agonists attenuate the ability of other G-protein-coupled receptors to initiate cell signaling; the underlying mechanisms entail phosphorylation of other receptors, G-proteins or AC, as well as alterations of the expression and/or activity of G proteins or AC itself (7, 15, 19, 34, 52).

The birth process is precede and accompanied by a sharp rise in circulating catecholamines (23). It would therefore be expected that β-AR responses should decrease during development as a consequence of agonist-induced desensitization. However, in many tissues, including the heart, lung and brown fat, the opposite is observed: β-AR sensitivity actually rises or even reaches a peak during this period (6, 20, 24, 28, 31, 38, 40, 46). Studies of the responses of newborns to prolonged β-agonist exposure suggest that β-AR desensitization and downregulation are not inherent properties, but rather are acquired during development (14, 38, 45, 46, 53, 55). Surprisingly, in neonatal rats, repeated administration of mixed β₁/β₂-agonists like isoproterenol, or selective β₂-agonists like terbutaline, elicit sensitization of β-AR/AC signaling instead of the anticipated desensitization (3, 14, 53, 54, 56). We recently found that this unique response to agonists represents heterologous effects downstream from the receptors themselves, thus affecting AC signaling mediated through receptors unrelated to the β-AR (53, 56). Heterologous, agonist-induced sensitization comprises at least three separate neonatal adaptations: induction of AC itself, enhanced AC catalytic activity, and enhanced coupling of
Gₛ-linked receptors to AC (53-56). Because of these mechanisms, repeated administration of isoproterenol or terbutaline to immature rats augments the AC response to β-AR stimulation, despite the fact that terbutaline (but not isoproterenol) downregulates β-ARs (2, 3).

The ability of β-agonists to induce signaling elements downstream from the β-AR in neonates raises an important question: do developing cells truly lack the ability to produce homologous desensitization, or is desensitization present, but masked by sensitization at post-receptor loci? The current study was designed to answer that question by evaluating β-AR/AC signaling in membranes prepared from the neonatal rat heart and liver within the first few hours after agonist administration. By comparing the immediate response after a single injection of β-agonist to that seen after repeated injections, we determined the extent to which homologous desensitization actually occurs, as compared to the extent to which it might be offset by longer-term, downstream adaptations. Homologous, β-AR-mediated effects on AC were contrasted with those evoked through the glucagon receptor, which shares signaling through Gₛ, and with those on the response to forskolin, which bypasses receptors but responds to association of Gₛ with AC (39). Newborn rats were compared to adults given the same treatments, so as to distinguish features that are essential to the immature pattern of agonist-induced sensitization from the mature pattern of desensitization. Finally, the role of specific β-AR subtypes were assessed in two ways. First, the effects of isoproterenol (β₁/β₂) were compared to those of terbutaline (β₂), as we previously found that these two agonists had differential effects on β-AR downregulation (2, 3). Second, effects on the heart were contrasted to those in the liver; these two tissues differ both in their relative expression of β-AR subtypes (β₁ predominant in heart, β₂ in liver), and in their ontogenetic patterns of receptor expression, since the heart acquires β-ARs during neonatal development (24) whereas the liver shows developmental decrements in β-AR expression (18).

METHODS

Animal treatments. Studies were carried out in accordance with the declaration of Helsinki
and with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. For neonatal experiments, primiparous, timed pregnant female Sprague-Dawley rats were shipped by climate-controlled truck (transit time, 12 h) and housed with free access to food and water. After birth, pups were randomized and redistributed to the nursing dams with litter sizes maintained at 10 pups; dams were reassigned daily to different litters to distribute any caretaking differences equally. Within each litter, equal numbers of males and females were assigned to each treatment group: daily s.c. injections on PN 2-5, of 1.25 mg/kg of \( l \)-isoproterenol hydrochloride, 10 mg/kg terbutaline hemisulfate or an equivalent volume (1 ml/kg) of vehicle (0.9% saline with 0.01% ascorbic acid). One, two and four hours after the first injection (acute treatment) or last injection (chronic treatment), hearts and livers were frozen in liquid nitrogen and stored at -45°C until assayed. Each treatment group contained equal numbers of males and females. For studies in adults, rats weighing approximately 280 g were given the same treatment regimens and the heart and a single lobe of the liver were taken after the first or last injection; experiments were restricted to males so as to avoid hormonal effects related to the estrus cycle. The isoproterenol and terbutaline treatment regimens used here elicit robust \( \beta \)-AR downregulation in adults (2).

*Adenylyl cyclase activity.* Tissues were thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in 39 volumes of ice-cold buffer containing 145 mM NaCl, 2 mM MgCl₂, and 20 mM Tris (pH 7.5), strained through several layers of cheesecloth when necessary to remove connective tissue and sedimented at 40,000 \( \times \) g for 15 min. The pellets were washed twice by resuspension (Polytron) in homogenization buffer followed by resedimentation, and were then dispersed with a homogenizer (smooth glass fitted with a Teflon pestle) to achieve a final protein concentration (27) of 0.5 – 1 mg/ml in a buffer consisting of 250 mM sucrose, 1 mM EGTA and 10 mM Tris (pH 7.4).

Aliquots of membrane preparation containing 25 - 50 µg protein were incubated for 30 min at 30°C with final concentrations of 100 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM ATP, 2 mM MgCl₂, 1 mg/ml bovine serum albumin, and a creatine phosphokinase-ATP-
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regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU/ml phosphocreatine kinase, with 10 µM GTP in a total volume of 250 µl. The enzymatic reaction was stopped by placing the samples in a 90 - 100°C water bath for 5 min, followed by sedimentation at 3000 × g for 15 min, and the supernatant solution was assayed for cAMP using radioimmunoassay kits. Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay period and was linear with membrane protein concentration; concentrations of cofactors were optimal and, in particular, the addition of higher concentrations of GTP produced no further augmentation of activity.

In addition to measuring basal AC activity, we also assessed the response to stimulants that act on different receptors but that share signal transduction through Gs: 100 µM l-isoproterenol, which stimulates β-ARs, and 3 µM glucagon, which stimulates glucagon receptors. We also assessed the response to the direct AC stimulant, 100 µM forskolin, which bypasses the need for receptor stimulation but which is optimized when Gs is associated with AC (39). Again, the concentrations of all these stimulants were optimal (1, 2, 53, 56).

Data analysis. Data are presented as means and standard errors. To establish treatment differences in AC activity, a global ANOVA (data log transformed because of heterogeneous variance) was first conducted across the in vivo treatment groups, age, tissue, number of injections, time after injection and all in vitro conditions under which AC was determined. The in vitro stimulant conditions were considered to be repeated measures, since each membrane preparation was used for the multiple types of AC determinations. As justified by significant interactions of treatment × age, treatment × tissue, treatment × injection number, and treatment × time (see Results), data were then subdivided to permit testing of individual treatments and AC measures that differed from control values; these were conducted by lower-order ANOVAs, followed, where appropriate, by Fisher’s Protected Least Significant Difference to identify specific time points at which the treated groups differed from the corresponding control. Tests of drug effects on body and tissue weights and on tissue membrane protein concentration were evaluated by similar procedures. Sex differences in drug responses were also evaluated, but only
for the neonatal treatment group, since adult studies were restricted to males. For all tests, significance for main treatment effects was assumed at \( p < 0.05 \); however, for interactions at \( p < 0.1 \), we also examined whether lower-order main effects were detectable after subdivision of the interactive variables (44).

For convenience, some data are presented as the percent change from control values but statistical differences were always established using the original data. Similarly, control values were combined for presentation across the three time points (1, 2, 4 h) for each age grouping, but statistical comparisons for treatment effects involved only the specific control groups matched for each treatment condition.

**Materials.** Animals were purchased from Zivic Laboratories (Pittsburgh, PA). Cyclic AMP radioimmunoassay kits were purchased from Amersham Pharmacia Biotech (Piscataway, NJ) and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

**RESULTS**

*Development of AC activity.* In keeping with earlier findings (31), cardiac AC activity did not develop monotonically from birth, but rather showed a distinct peak during the first postnatal week, before declining to lower values in adulthood (Fig. 1, top panel). Superimposed on this basic pattern, across all ages tested (PN2, PN5, adult), isoproterenol evoked significant AC stimulation (\( p < 0.0001 \)) of about 2-3-fold relative to basal activity; direct AC stimulation by forskolin resulted in massive increases in activity (more than 10-fold above basal AC, \( p < 0.0001 \)), again following the basic developmental pattern of a peak on PN5. Glucagon produced a smaller stimulation in the neonate (10% increase), rising to a 2-fold increase in adulthood. The pattern for glucagon, a monotonic rise from neonatal to adult values, was distinct from that for basal activity, isoproterenol-stimulated activity, or forskolin-stimulated activity (\( p < 0.0001 \) for interaction of age \( \times \) stimulant). In addition to changes in AC activity with increasing age, there were distinct differences between males and females on PN2 and PN5, with activities in females uniformly higher (main effect of sex). The sex difference did not reflect a difference in the total
concentration of membrane proteins, which showed no sex differences (data not shown).

The liver displayed a distinctly different ontogenetic pattern, with a decrease in basal, isoproterenol-stimulated and forskolin-stimulated AC activities between PN2 and adulthood (Fig. 1, bottom panel). Overall, the forskolin-stimulated activity was much lower in the liver than in the heart (10-fold increase over basal as compared to a 30-fold increase). Again, however, the response to glucagon was distinctly different, with responses sustained into adulthood, in keeping with the ontogenetic rise in glucagon receptor concentrations (4); indeed, given the decline in basal activity with age, the maintenance of the effect of glucagon resulted in a rise from 2-fold stimulation over basal values in the neonate, to 5-fold stimulation in adulthood. As in the heart, neonatal rats showed a significant sex difference in liver AC, with uniformly higher values in females.

General effects of β-agonist treatment. Neither isoproterenol nor terbutaline administration to neonatal rats had any significant effect on body weight, tissue weight or membrane protein concentrations (data not shown). In contrast, in adults (n=16-18 per group), repeated isoproterenol treatment had a deleterious effect on body weight (340 ± 4 g in controls, 325 ± 4 g in the isoproterenol group, p < 0.003), and both chronic isoproterenol and terbutaline increased the heart weight: 968 ± 10 mg in controls, 1195 ± 25 mg in the isoproterenol group (p < 0.0001), 1060 ± 18 mg in the terbutaline group (p < 0.0007). However, only the chronic isoproterenol treatment elicited the decrease in membrane protein concentration that is characteristic of cell enlargement (2): 66 ± 2 mg/g in controls; 53 ± 1 mg/g in the isoproterenol group (p < 0.0001); 64 ± 1 mg/g in the terbutaline group (NS). A single injection of either of the β-agonists did not alter any of these parameters in adult rats (data not shown).

Global statistical analysis of the effects of isoproterenol or terbutaline on measures of AC activity indicated a significant main effect of drug treatment (p < 0.0001) that differed according to age, the number of injections, the time after injection, tissue, and the specific AC measure (Table 1). The interactions were maintained in comparing either β-agonist-treatment to control groups, as well as when comparing the β-agonists to each other. In light of the highly interactive
effects, we subdivided the data for presentation along logical lines: neonates versus adults, heart versus liver, effects on the different AC stimulants. We also performed a global analysis including the sex variable in neonates and identified a significant contribution of sex to treatment effects (see Results). However, when data were subdivided according to the interactions with the other variables, all main effects of β-agonists were present in both sexes in nearly equivalent magnitudes. Accordingly, we did not subdivide the two sexes for presentation of treatment effects, although we maintained the sex variable in the statistical analyses.

Effects on basal AC activity. In the adult heart, administration of a single injection of isoproterenol or terbutaline had a significant effect on basal AC activity, measured in the presence of GTP (Fig. 2, upper left panel). With either β-agonist, basal cardiac AC showed elevations of about 20%, persisting through at least four hours post-injection. By the fourth time that the drugs were administered, however, there were adaptive changes offsetting the increases: isoproterenol evoked significant decrements in basal AC and terbutaline had little or no effect. In contrast to the results seen in adult heart, neonates showed much smaller effects of β-agonists on basal AC activity (Fig. 2, upper right panel). A single injection of isoproterenol or terbutaline evoked acute increments of only 5-10%; again, adaptive changes were evident with repeated drug administration, as the stimulatory effects were lost by the fourth injection, but in the neonates, unlike the adults, there was no significant decrement in basal activity with the fourth injection of isoproterenol.

The adult liver showed different reactivity from that seen in the heart (Fig. 2, bottom left panel). Basal AC showed only small, nonsignificant increases in the four hour period after the first injection of isoproterenol or terbutaline. With the fourth injection of either agonist, there was again, little change in basal AC in the ensuing four hours. When the single treatment was given to neonates, however, there were substantial decrements in basal AC for the first 2 hours (Fig. 2, lower right panel). With repeated β-agonist administration to neonates, basal liver AC showed the same decrements but the effect of terbutaline lasted through at least 4 hours.

Given the significant changes in basal AC evoked by β-agonists, we evaluated the response
to stimulants as the proportional change over basal activity. Under these conditions, homologous desensitization of the β-AR response represents a decrease in the proportional response to isoproterenol without comparable change in the response to glucagon. Heterologous desensitization requires an equivalent reduction in the proportional response to both stimulants.

**Effects on the cardiac response to AC stimulants.** The *in vitro* response to β-AR stimulation was evaluated by addition of isoproterenol to the assay medium. In the adult heart, a single injection of isoproterenol evoked significant (p < 0.05) desensitization of the β-AR response (Fig. 3, upper left panel), as found earlier (53); terbutaline did not cause desensitization (p > 0.8), so that overall significance was lost when determined across both of the β-agonist treatments (p < 0.11). By the fourth injection, isoproterenol treatment evoked robust desensitization, with loss of up to 25% of the response, but terbutaline did not evoke desensitization. In contrast to adults, neonates showed no loss of the cardiac AC response to β-AR stimulation after either the first or fourth injection of isoproterenol or terbutaline (Fig. 3, upper right panel).

For adult cardiac responses to glucagon, a single injection of isoproterenol or terbutaline did not evoke significant changes (Fig. 3, middle left panel). However, just as for the β-AR response, the ability of glucagon to stimulate AC was robustly reduced by the fourth injection of isoproterenol; accordingly, the parallel decline of both responses represents heterologous desensitization. As before, terbutaline was ineffective in evoking desensitization. When the same drug treatments were given to neonatal rats (Fig. 3, middle panel), the glucagon response was unaffected after either the first or fourth injection.

The changes in basal AC and the heterologous changes in the responses to isoproterenol and glucagon suggested that alterations were occurring at the level of G-protein interactions with AC. Accordingly, we also tested the AC response to forskolin, which acts without the intervention of receptors, but is affected by the association of G-proteins with AC. In the adult heart, a single injection of isoproterenol or terbutaline evoked a robust, but transient, increase in the AC response to forskolin (Fig. 3, bottom left panel). With repeated isoproterenol administration, this response was no longer evident. On the other hand, with repeated administration of terbutaline,
we still observed stimulation of the forskolin response. When the same β-agonists were given to neonatal rats, just as in adults, the forskolin response of cardiac AC showed transient promotion after a single injection of either isoproterenol or terbutaline (Fig. 3, lower right panel). After the fourth injection, the response to isoproterenol was lost and the effect of terbutaline was diminished, but still statistically significant.

**Effects on the hepatic response to AC stimulants.** In stark contrast to the effects seen in the heart, administration of isoproterenol to adult rats failed to elicit significant changes in the response of hepatic AC to isoproterenol (Fig. 4, upper left panel) or glucagon (Fig. 4, middle left panel) *in vitro*, regardless of whether the animals received a single injection or multiple injections. Terbutaline treatment similarly did not alter the receptor-mediated responses. In this tissue, unlike the heart, neonates were far more sensitive than adults: a single injection of isoproterenol or terbutaline evoked immediate desensitization of the hepatic AC response to β-AR stimulation, with a larger net effect of terbutaline (Fig. 4, upper right panel). Repeated administration of either agonist elicited even larger decrements in the β-AR/AC response, reaching 40% desensitization by isoproterenol administration and 60% for terbutaline. On the other hand, the neonates showed *sensitization* of the hepatic response to glucagon after treatment with either isoproterenol or terbutaline (Fig. 4, middle right panel). Repeated administration of the β-AR agonists augmented the effect, with terbutaline producing a greater enhancement than isoproterenol. Thus, unlike the situation in the adult heart, where we observed heterologous desensitization by β-AR agonists (parallel reductions in the response to isoproterenol and glucagon), the β-AR desensitization seen in neonatal liver was homologous (limited to the isoproterenol response).

Both isoproterenol and terbutaline had significant effects on the hepatic forskolin/AC response. In adults, a single injection of terbutaline evoked increases in the forskolin response (Fig. 4, lower left panel); terbutaline was more effective than isoproterenol, whereas in the heart, isoproterenol had been more effective. Repeated β-agonist administration showed an augmented response, unlike the situation in the heart. In neonates, a single injection of isoproterenol or
terbutaline had only minor, nonsignificant stimulatory effects on the hepatic AC response to forskolin (Fig. 4, lower right panel). With repeated treatment, however, the responses were significantly augmented; again, terbutaline was more effective than isoproterenol.

*Sex differences in the neonatal response to β-agonists.* In light of the sex differences seen for development of basal AC and AC responses to stimulants in control animals (Fig. 1), we also examined our results to see if there were differential effects of isoproterenol or terbutaline treatment on cardiac or hepatic AC responses in neonates. Across all four AC measures, we found significant interactions of treatment × sex, as well as treatment × sex × other variables (Table 2); again, these interactions were present across all three treatment groups and between each pair of treatments. However, after subdivision of the results into the individual tissues, treatment regimens, and AC stimulant categories described above, in only three instances were there statistically significant and clear distinctions between the responses to β-AR agonist treatments in males and females (data not shown). For basal AC activity after the fourth injection of isoproterenol, females showed a significant decrease, whereas males did not (treatment × sex, p < 0.03). For the hepatic response to glucagon after the first injection of either isoproterenol or terbutaline, females showed a quicker time course than males, with a more rapid increase in the response and a more rapid decline from the peak effect (treatment × sex × time, p < 0.0003); however, both sexes showed the main effect of an overall increase in the glucagon response. For the cardiac response to forskolin after the first injection of β-agonist, males showed a bigger increase than females (treatment × sex, p < 0.0009), but again, both sexes did show a significant overall effect in the same direction.
DISCUSSION

Unlike the situation in the adult, prolonged β-AR stimulation in neonates does not desensitize receptor-mediated signaling, and instead, signaling is maintained or even enhanced through heterologous mechanisms downstream from the receptor (14, 38, 45, 46, 53, 55). Here, we found distinct tissue differences in the ability of developing cells to desensitize, and identified a combination of unique processes that dictate the balance between agonist-induced desensitization and sensitization. These events include differential expression of receptor and AC subtypes, as well as adaptive changes to repetitive stimulation that occur only in the immature organism. Our findings indicate a clear-cut disparity between regulatory responses in developing heart as compared to liver, tissues that differ both in the predominant β-AR subtype and in their patterns of receptor expression; whereas the neonatal heart possesses a 2:1 majority of β₁-receptors (43), the immature liver has almost exclusively the β₂ subtype (2), and whereas receptor numbers are maintained or increase with age in the heart (22, 24), they decline precipitously in the liver (18). Changes in β-AR concentrations are almost certainly responsible for the tissue-specific differences we saw in the patterns of β-AR-mediated AC responses in membrane preparations from control animals: the stimulatory effect of isoproterenol increased between birth and adulthood in the heart, but declined in the liver, whereas responses to glucagon increased with age in both tissues. Superimposed on these receptor-driven differences, AC subtypes that determine the balance between desensitization and sensitization change with development. Both heart and liver express AC types V and VI (34, 35), which have a phosphorylation site for protein kinase A that leads to heterologous desensitization (34). However, type VI is sensitized by βγ-subunits released from the trimeric G-protein when agonists are associated with G-protein-coupled receptors (48). The relative expression of AC isoforms changes during development and with β-agonist treatment (11, 13, 54), and as seen from the results in control animals here, can readily influence the proportional response to AC stimulants, such as forskolin, in different tissues. Accordingly, we will first examine the effects of isoproterenol and terbutaline treatments separately for heart and liver, and then discuss what factors are most likely to
contribute to the variant responses between the two.

With the first injection of isoproterenol to adult rats, we readily observed short-term activation of $G_s$, evidenced by increases in basal and forskolin-stimulated AC activity, and the effect was equally apparent for a mixed $\beta_1/\beta_2$ agonist (isoproterenol) and for a $\beta_2$-selective agonist (terbutaline). Accordingly, despite disparities in the proportion of receptor subtypes, the higher coupling efficiency of $\beta_2$-ARs (16, 26), as well as the undoubtedly more prolonged actions of terbutaline, lead to equivalent initial activation of cell signaling. In keeping with earlier results (53), this effect was accompanied by a small degree of homologous desensitization (decreased *in vitro* isoproterenol response without desensitization of the glucagon response) evoked by the isoproterenol treatment but not by terbutaline, a first indicator of subtype-related differences in the response pattern. These disparities became a major feature with repeated agonist administration, where isoproterenol elicited heterologous desensitization (loss of both the AC responses to a $\beta$-AR agonist and glucagon) but terbutaline did not. Accordingly, there are two distinct phases of the adult response to $\beta$-AR activation: with the first exposure, $G_s$ is activated and $\beta$-ARs show homologous desensitization, whereas with repeated administration, heterologous desensitization emerges. Comparing these findings to those obtained in neonatal rats, we found that acute isoproterenol or terbutaline treatment also activated $G_s$, as evidenced by increases in basal and forskolin-stimulated AC activity; most notably, however, we did not observe homologous desensitization after the first injection of isoproterenol, nor did we see heterologous desensitization after the fourth injection. Despite the neonatal resistance of receptor-mediated signaling to agonist-induced desensitization, we did obtain some evidence for inhibitory effects on G-protein function, as the acute stimulatory effect on the forskolin response was lost (isoproterenol) or diminished (terbutaline) after repeated $\beta$-agonist injections. The preservation of receptor signaling in the face of decrements in G-protein function probably reflects the vast excess of G-proteins relative to receptors or AC (33). In contrast, receptor-mediated signaling is directly responsive to changes in AC itself (12, 13); in keeping with this view, we found that, with neonatal $\beta$-agonist treatment, AC induction 24 h after the fourth
injection produces an augmented AC response to isoproterenol and glucagon (3, 56).

Conceivably, some of the age-related differences in desensitization could reflect the proportion of β-AR subtypes. The immature heart has a higher percentage of β₂-ARs than the adult (2, 43) and since we found that, in the adult, terbutaline was less effective in eliciting β-AR desensitization, it is logical to suppose that the β₂-subtype may protect the neonate from desensitization. If that were the sole factor, then the liver, which contains almost exclusively β₂-ARs regardless of age, should likewise show resistance to desensitization. Indeed, AC in the adult liver maintained its responsiveness to isoproterenol and glucagon in the face of repeated administration of either of the β-AR agonists. The failure to evoke desensitization was not simply due to a failure of cell stimulation due to the low β-AR concentrations in adult liver, as we found robust activation of forskolin-stimulated AC activity. However, when we examined AC responses in the neonatal liver, we were surprised to find robust, homologous desensitization, regardless of whether animals received one or four injections of agonist. Since the neonatal liver also contains predominantly β₂-ARs, this makes it highly unlikely that receptor subtype is the major determinant of the presence or absence of desensitization. In fact, in this case, terbutaline elicited greater desensitization than did isoproterenol, indicating that, in the liver, it is indeed stimulation of the β₂-ARs, not a small population of β₁-ARs, that leads to the loss of response. In contrast to β-AR desensitization, the neonatal hepatic response to glucagon was not only maintained, but actually showed sensitization, again likely reflective of the induction of downstream signaling elements (47); in support of this view, the hepatic response to forskolin was also enhanced to a much greater extent after four injections in neonates.

Our results thus indicate that neonates and adults differ in their immediate responses to β-AR activation, and to a greater extent, in their long-term adaptations to prolonged stimulation. The results in the liver show that immature cells are inherently capable of homologous desensitization in response to β-agonists; at the same time, the absence of desensitization in the neonatal heart indicates a fundamental difference between tissues that is most likely to reside in adaptive changes in the expression and activity of signaling proteins downstream from the
receptor. We have already shown that, uniquely in the neonatal heart, isoproterenol administration elicits an increase in $G_s$-mediated responses, suppression of $G_i$ expression, induction of AC itself, and shifts in AC isoforms (53-56), all of which are likely to assist in the resistance to desensitization. Although some of these mechanisms are present in the neonatal liver (2, 3, 47), there must be basic differences, at the level of G-protein function and AC, that produce the greater sensitivity of this tissue to agonist-induced desensitization as seen here. The ability of G-protein activation to produce heterologous sensitization of AC is highly dependent on AC subtype and the proportions of different G-proteins within the cell (51); given that heterologous sensitization of AC is the major mechanism by which neonatal tissues preserve their responsiveness, it would be worthwhile to examine the hepatic patterns of G-protein and AC subtype expression in response to $\beta$-agonists, as has been done for the heart (53-55). Recruitment of signaling proteins to elicit heterologous sensitization, unlike the situation for desensitization, could require a specific receptor subtype. $\beta_2$-AR-mediated activation of protein kinase A is compartmentalized (21), which could direct phosphorylation away from those elements eliciting desensitization and towards those promoting sensitization. In the neonatal liver, which highly expresses $\beta_2$-ARs, both isoproterenol and terbutaline sensitized AC to glucagon or forskolin, but the sensitization by terbutaline ($\beta_2$-agonist) was greater. Furthermore, sensitization in the neonatal liver was greater than that in the neonatal heart, which expresses predominantly $\beta_1$-ARs. Finally, even in the adult heart, repeated treatment with the $\beta_2$-AR agonist, terbutaline, was able to sustain the cardiac AC response to forskolin, whereas the mixed agonist, isoproterenol, was not. The greater ability of $\beta_2$-ARs to elicit heterologous sensitization of AC is also consistent with their more efficient coupling to AC (16, 26).

In adults, one additional mechanism contributes to tissue differences in the ability to desensitize $\beta$-AR/AC signaling: cellular enlargement reduces the surface-to-volume ratio, diluting all membrane proteins and thus reducing the concentrations of $\beta$-ARs, G-proteins and AC relative to cell volume (2, 56). Repeated isoproterenol administration elicits cellular hypertrophy in the heart but not in the liver (2, 47, 56), and this type of heterologous
desensitization accounts for as much as 50% of the loss of cardiac signaling in the adult (2, 56). In the present study, dilution of cardiac membrane proteins was evident from the significant reduction in membrane protein concentrations in adults given four injections of isoproterenol. In contrast, we saw no hypertrophy with either isoproterenol or terbutaline in the neonatal heart, so that, in the immature organism, the tissue differences in agonist-induced sensitization or desensitization clearly do not reflect heterologous dilution of membrane proteins.

Our results point to another area for future exploration: the potential for sex differences in the neonatal response to β-agonist challenge. We found that AC activity and responsiveness were higher in females, and we identified small but significant differences between male and female responses to β-AR challenge. It is thus possible that sex contributes to the basic response to, and adaptive changes elicited by, β-agonist treatments during development. Sex-related differences have been noted in β-AR responses in cardiac myocytes isolated from mature rats (50) but, to our knowledge, this is the first report of such sex differences in neonatal development.

In conclusion, β-AR agonists are incapable of eliciting desensitization of neonatal cardiac β-AR/AC signaling, as we found neither homologous nor heterologous desensitization after acute or chronic treatment with isoproterenol or terbutaline. On the other hand, the same treatments elicited robust, homologous desensitization of signaling in the liver, although even in that tissue, subsequent AC induction eventually masks the desensitization (47). Accordingly, the resistance of neonatal tissues to loss of β-AR signaling represents active, adaptive processes at signaling steps downstream from the β-AR, rather than an inherent inability to desensitize. Even in the liver, where short-term desensitization was elicited, the loss of β-AR responses was accompanied by augmentation of glucagon-mediated responses, which provide similar metabolic effects on gluconeogenesis and glycogenolysis. These unique neonatal adaptations are thus likely to provide for maintenance of receptor-mediated signaling in the face of the high catecholamine levels in the period surrounding birth (23). On the other hand, β-AR agonists, particularly terbutaline, are commonly used to arrest preterm labor, with treatment often extending for many
weeks (10, 37). In this case, the failure to elicit β-AR desensitization may contribute to adverse outcomes. β-AR stimulation plays a dual role in cardiac cell replication and differentiation, initially maintaining replication (41, 57), and subsequently terminating replication and initiating differentiation (8, 42); excessive stimulation elicits apoptosis (9). Accordingly, β-AR excitation in the immature organism, unrestrained by desensitization, may be responsible for the cardiac structural and physiological anomalies, and the alterations in hepatic glucose metabolism, that have been noted in neonates exposed to tocolytic β-AR agonists (5, 25, 36).

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**REFERENCES**


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TABLE 1. GLOBAL STATISTICAL ANALYSIS

<table>
<thead>
<tr>
<th>Interaction</th>
<th>All Treatments</th>
<th>Control vs. Isoproterenol</th>
<th>Control vs. Terbutaline</th>
<th>Isoproterenol vs. Terbutaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rx (main effect)</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>NS</td>
<td>p &lt; 0.0002</td>
</tr>
<tr>
<td>Rx × Injection</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.07</td>
</tr>
<tr>
<td>Rx × Time</td>
<td>p &lt; 0.05</td>
<td>NS</td>
<td>NS</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>Rx × Age</td>
<td>p &lt; 0.0001</td>
<td>NS</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Rx × Tissue</td>
<td>p &lt; 0.0001</td>
<td>NS</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Rx × Measure</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Rx × Injection × Age</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0002</td>
<td>NS</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Rx × Injection × Tissue</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>NS</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Rx × Time × Tissue</td>
<td>p &lt; 0.006</td>
<td>p &lt; 0.002</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Rx × Age × Tissue</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>NS</td>
</tr>
<tr>
<td>Rx × Injection × Measure</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0007</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
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<tr>
<td>Rx × Time × Measure</td>
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<td>p &lt; 0.002</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.04</td>
</tr>
<tr>
<td>Rx × Age × Measure</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Rx × Tissue × Measure</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Rx × Injection × Time × Age</td>
<td>NS</td>
<td>NS</td>
<td>p &lt; 0.09</td>
<td>NS</td>
</tr>
<tr>
<td>Rx × Injection × Time × Tissue</td>
<td>p &lt; 0.04</td>
<td>NS</td>
<td>p &lt; 0.04</td>
<td>p &lt; 0.03</td>
</tr>
<tr>
<td>Rx × Injection × Age × Tissue</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>NS</td>
<td>p &lt; 0.0003</td>
</tr>
<tr>
<td>Rx × Injection × Time × Measure</td>
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<td>p &lt; 0.0001</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.03</td>
</tr>
<tr>
<td>Rx × Injection × Age × Measure</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.008</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Rx × Injection × Tissue × Measure</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Rx × Time × Age × Measure</td>
<td>p &lt; 0.008</td>
<td>NS</td>
<td>p &lt; 0.003</td>
<td>p &lt; 0.04</td>
</tr>
<tr>
<td>Rx × Age × Tissue × Measure</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Rx × Injection × Time × Age × Measure</td>
<td>p &lt; 0.02</td>
<td>NS</td>
<td>p &lt; 0.007</td>
<td>p &lt; 0.08</td>
</tr>
<tr>
<td>Rx × Injection × Time × Tissue × Measure</td>
<td>p &lt; 0.006</td>
<td>NS</td>
<td>p &lt; 0.004</td>
<td>p &lt; 0.04</td>
</tr>
<tr>
<td>Rx × Injection × Age × Tissue × Measure</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Rx × Time × Age × Tissue × Measure</td>
<td>p &lt; 0.04</td>
<td>NS</td>
<td>p &lt; 0.09</td>
<td>p &lt; 0.02</td>
</tr>
</tbody>
</table>

Values in the table are shown only for interactions between treatment and other variables; treatment interactions that are not shown in the table were not significant (NS). Rx = treatment; Injection = injection number (first or fourth); Time = interval after the injection (1, 2, 4 h); Age = neonates vs. adults; Tissue = heart vs. liver; Measure = the four different AC measures (basal, isoproterenol, glucagon, forskolin).
TABLE 2. SEX-DEPENDENT EFFECTS IN NEONATES

<table>
<thead>
<tr>
<th>Interaction</th>
<th>All Treatments</th>
<th>Control vs. Isoproterenol</th>
<th>Control vs. Terbutaline</th>
<th>Isoproterenol vs. Terbutaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rx × Sex</td>
<td>p &lt; 0.0005</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Rx × Sex × Time</td>
<td>p &lt; 0.006</td>
<td>p &lt; 0.002</td>
<td>NS</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>Rx × Sex × Tissue</td>
<td>p &lt; 0.02</td>
<td>p &lt; 0.04</td>
<td>NS</td>
<td>p &lt; 0.006</td>
</tr>
<tr>
<td>Rx × Sex × Measure</td>
<td>p &lt; 0.002</td>
<td>p &lt; 0.009</td>
<td>p &lt; 0.0006</td>
<td>NS</td>
</tr>
<tr>
<td>Rx × Sex × Injection × Time</td>
<td>NS</td>
<td>p &lt; 0.09</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Rx × Sex × Injection × Measure</td>
<td>p &lt; 0.06</td>
<td>p &lt; 0.06</td>
<td>NS</td>
<td>p &lt; 0.09</td>
</tr>
<tr>
<td>Rx × Sex × Time × Measure</td>
<td>p &lt; 0.004</td>
<td>p &lt; 0.002</td>
<td>p &lt; 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Rx × Sex × Injection × Time × Tissue</td>
<td>NS</td>
<td>p &lt; 0.06</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Rx × Sex × Injection × Tissue × Measure</td>
<td>NS</td>
<td>NS</td>
<td>p &lt; 0.08</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Rx × Sex × Injection × Time × Measure</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0009</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Rx × Sex × Time × Tissue × Measure</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>p &lt; 0.08</td>
</tr>
<tr>
<td>Rx × Sex × Injection × Time × Tissue × Measure</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.06</td>
<td>p &lt; 0.02</td>
<td>p &lt; 0.06</td>
</tr>
</tbody>
</table>

Values in the table are shown only for interactions between treatment × sex × other variables; treatment × sex interactions that are not shown in the table were not significant (NS). Rx = treatment; Injection = injection number (first or fourth); Time = interval after the injection (1, 2, 4 h); Age = neonates vs. adults; Tissue = heart vs. liver; Measure = the four different AC measures (basal, isoproterenol, glucagon, forskolin).
FIGURE LEGENDS

**Figure 1.** Development of AC activity in control heart and liver. Data represents means and standard errors obtained from 18-36 animals in each group. Note the different ordinate scale for forskolin in the upper panel. For each tissue, ANOVA across all AC measures appears at the top of the panel, with subdivision by measure at the bottom of the panels. Across both tissues, ANOVA indicates significant main effects of age (p < 0.0001) and tissue (p < 0.0001) as well as interactions of age × tissue, age × stimulant, tissue × stimulant, and age × tissue × stimulant (all at p < 0.0001).

**Figure 2.** Effects of isoproterenol or terbutaline treatment on basal AC activity in adult and neonatal heart (top panels) and liver (bottom panels). Data represent means and standard errors obtained from 8-24 animals for each group, presented as the percent change from control values, which appear in Fig. 1. ANOVA across both injection paradigms and all time points appears at the top of each panel, with subdivision for each injection paradigm at the bottom. In addition, ANOVA across ages and tissues indicates a significant difference in the effects seen in adults compared to neonates (treatment × age, p < 0.0001) and between heart and liver (treatment × tissue, p < 0.0001). Significance of individual time points (asterisks) was determined only where there was a significant interaction of treatment × time after subdivision of the data into separate treatment paradigms (neonatal liver); asterisks denote individually significant values. Abbreviations: Rx = treatment, Con = control, Iso = isoproterenol, Ter = terbutaline.

**Figure 3.** Effects of isoproterenol or terbutaline treatment on the response to AC stimulants in adult and neonatal heart. Data represent means and standard errors obtained from 8-24 animals for each group, presented as the percent change from control values; responses were defined as the proportional increase in AC activity over basal values. ANOVA across both injection paradigms and all time points appears at the top of each panel, with subdivision for each injection paradigm at the bottom. In addition, ANOVA across ages and stimulants indicates a significant difference in the effects seen in adults compared to neonates (treatment × age, p < 0.0002) and among the different stimulants (treatment × stimulant, p < 0.0001). Significance of
individual time points (asterisks) was determined only where there was a significant interaction of treatment × time after subdivision of the data into separate treatment paradigms. Abbreviations: Rx = treatment, Con = control, Iso = isoproterenol, Ter = terbutaline.

**Figure 4.** Effects of isoproterenol or terbutaline treatment on the response to AC stimulants in adult and neonatal liver. Data represent means and standard errors obtained from 8-24 animals for each group, presented as the percent change from control values; responses were defined as the proportional increase in AC activity over basal values. ANOVA across both injection paradigms and all time points appears at the top of each panel, with subdivision for each injection paradigm at the bottom. In addition, ANOVA across ages and stimulants indicates a significant difference in the effects seen in adults compared to neonates (treatment × age, p < 0.0001) and among the different stimulants (treatment × stimulant, p < 0.0001). Significance of individual time points (asterisks) was determined only where there was a significant interaction of treatment × time after subdivision of the data into separate treatment paradigms. Abbreviations: Rx = treatment, Con = control, Iso = isoproterenol, Ter = terbutaline.
**Figure 1**

**Heart**

ANOVA: Age, p < 0.0001; Age x Stimulant, p < 0.0001; Sex, p < 0.0001; Sex x Stimulant, p < 0.0001; Sex x Age x Stimulant, p < 0.0077

**Liver**

ANOVA: Age, p < 0.0001; Age x Stimulant, p < 0.0001; Sex, p < 0.0001; Sex x Stimulant, p < 0.02; Sex x Age x Stimulant, p < 0.08
Figure 2

**Adult Heart - Basal AC Activity**

ANOVA: Rx, p < 0.003; Rx x Injection number, p < 0.0001

First Injection

Rx, p < 0.004;
Con < Iso, p < 0.006
Con < Ter, p < 0.0006

Fourth Injection

Rx, p < 0.0001;
Con > Iso, p < 0.0001
Con > Ter, p < 0.0002

**Neonatal Heart - Basal AC Activity**

ANOVA: Rx x Injection number, p < 0.005

First Injection

Rx, p < 0.003;
Con < Iso, p < 0.05

Fourth Injection

Rx, p < 0.0001;
Con > Iso, p < 0.0001
Con > Ter, p < 0.002

**Adult Liver - Basal AC Activity**

ANOVA: Rx x Injection number, p < 0.08; Rx x Time, p < 0.05

**Neonatal Liver - Basal AC Activity**

ANOVA: Rx, p < 0.0001; Rx x Injection number, p < 0.04; Rx x Time, p < 0.0001; Rx x Injection number x Time, p < 0.02
**Figure 3**

**Adult Heart - Isoproterenol Response**

- ANOVA: Rx, p < 0.0001; Rx x Injection number, p < 0.004

**Neonatal Heart - Isoproterenol Response**

- ANOVA: Rx, p < 0.03

**Adult Heart - Glucagon Response**

- ANOVA: Rx, p < 0.0001; Rx x Injection number, p < 0.0001

**Neonatal Heart - Glucagon Response**

- ANOVA: NS

**Adult Heart - Forskolin Response**

- ANOVA: Rx, p < 0.007; Rx x Injection number, p < 0.003

**Neonatal Heart - Forskolin Response**

- ANOVA: Rx, p < 0.0001; Rx x Injection number, p < 0.0001; Rx x Time, p < 0.0001; Rx x Injection number x Time, p < 0.0001

- Rx, p < 0.002; Rx x Time, p < 0.01; Rx x Injection number x Time, p < 0.0001

- Rx, p < 0.005; Rx x Time, p < 0.01; Rx x Injection number x Time, p < 0.0001