The bile acid taurocholate impairs rat cardiomyocyte function: a proposed mechanism for intra-uterine fetal death in obstetric cholestasis

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ABSTRACT

Obstetric cholestasis is a liver disease of pregnancy that can be complicated by sudden, hitherto unexplained, intra-uterine fetal death. Because intra-uterine death occurs suddenly, and because fetal heart rate abnormalities have been reported in obstetric cholestasis, we hypothesized that intra-uterine death is caused by impaired fetal cardiomyocyte function, resulting in fetal cardiac arrest. Obstetric cholestasis is associated with raised levels of maternal and fetal serum bile acids, and we propose that these may alter cardiomyocyte function. It was not possible to investigate the effects of bile acids on the intact human fetal heart at a cellular level. Therefore we used the closest available model of fetal myocardium at term: a primary culture of neonatal rat cardiomyocytes in which cells beat synchronously and develop pacemaker activity. The effect of the primary bile acid taurocholate (0.3 mM and 3 mM) on cultures of single cardiomyocytes, each with its own independent rate of contraction, was a reversible decrease in the rate of contraction and in the proportion of beating cells (P < 0.001). Addition of taurocholate to a network of synchronously beating cells caused a similar decrease in the rate of contraction. Furthermore, the integrity of the network was destroyed, and cells ceased to beat synchronously. Taurocholate also resulted in altered calcium dynamics and loss of synchronous beating. These data suggest that raised levels of the bile acid taurocholate in the fetal serum in obstetric cholestasis may result in the development of a fetal dysrhythmia and in sudden intra-uterine death.

INTRODUCTION

Obstetric cholestasis (OC) is a liver disorder of pregnancy with serious consequences for the mother and fetus [1–4]. The most characteristic maternal feature is generalized pruritus, which becomes more severe with advancing gestation. OC can cause fetal distress, spontaneous premature delivery and unexplained third-trimester intra-uterine death [1–4], which usually occurs after 37 weeks’ gestation. Abnormal maternal liver function tests are necessary to make the diagnosis. In particular, the serum total bile acid concentration is raised compared with normal pregnancy [5–8]. In OC the serum bile acid concentrations can increase 100-fold, but

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Abbreviations: OC, obstetric cholestasis; TC, taurocholate.

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return to normal within 1 week of delivery. In addition, the increase in cholic acid is much more marked than that in chenodeoxycholic acid and deoxycholic acid [5,6,9]. Raised maternal bile acid levels have been shown to be associated with fetal distress [7,8], and several authors have postulated that raised maternal and fetal bile acid levels are the cause of the adverse fetal consequences of OC [6,7,9,12].

The mechanism of intra-uterine death in OC pregnancies is poorly understood. It is thought to occur suddenly, as there is no evidence of preceding intra-uterine growth restriction or utero-placental insufficiency, and the fetal autopsy is normal. Some studies have demonstrated an abnormal fetal heart rate (≤ 100 or ≥ 180 beats/min) in OC pregnancies [8,12], and severe fetal bradycardia (< 100 beats/min) was noted in 16% of neonates in one study [1].

Because intra-uterine death in OC occurs suddenly, and because an abnormal fetal heart rate has been demonstrated, we hypothesized that raised levels of bile acids cause impaired fetal cardiomyocyte function, resulting in fetal cardiac arrest. It is not possible to investigate the effects of bile acids on the intact human fetal heart at a cellular level. Therefore we used the closest available model of fetal myocardium at term: a primary culture of neonatal rat cardiomyocytes in which cells beat synchronously [13] and develop pacemaker activity [14]. Neonatal rat cardiomyocytes provide a unique in vitro model for studying the rhythm, intracellular calcium dynamics and cell-to-cell interactions of cardiomyocytes [15–17]. Cardiomyocytes can be grown as primary cultures of single cells, each with its own independent rate of contraction, or as a network of cells that beat synchronously. When cultured as a network, the early culture consists of single myocytes which exhibit an unsynchronized beating pattern. When any two spontaneously beating neonatal myocytes establish contact, they synchronize. Intercellular coupling via gap junction formation is crucial for this synchronization [18]. Subsequently the cells proliferate, migrate and assemble into a monolayer network that beats synchronously [14].

The aims of the present study were to investigate whether the tauroconjugated primary bile acid taurocholate (TC) alters the rate of contraction, the synchronization of contraction or the Ca^{2+} dynamics of rat neonatal cardiomyocytes.

METHODS

Preparation of a primary culture of neonatal rat cardiomyocytes
Heart cells were obtained as described by Iwaki et al. [19]. Cells were kept in Dulbecco’s modified Eagle’s minimum essential medium containing 5% (v/v) fetal calf serum (Gibco), 200 µg/ml streptomycin, 200 units/ml penicillin and non-essential amino acids (all from Gibco, Paisley, Scotland, U.K.). Geniticin (G418; Gibco) at 100 µg/ml was added to inhibit fibroblast growth. Cells were maintained at 37 °C, in an atmosphere of humidified air plus 5% CO_{2}. Cells were used 2 days (discrete cells) and 5 days (network of cells) after plating. The cardiomyocytes were cultured on Petri dishes or glass coverslips. The study protocol is summarized in Figure 1.

Investigation of the effects of TC on the rate of contraction and on the proportion of contracting cardiomyocytes
TC was added to give a final concentration of 0.3 mM or 3.0 mM in the culture medium of single cardiomyocytes and of the network of synchronously beating cells.

For cultures of single cells, the rate of contraction was calculated for 15 s in individual cells, and expressed as beats/min. All measurements were made by a single observer, to avoid operator bias. For the network of cardiomyocytes, the rate of contraction was measured in different parts of the network in the same way as for single cardiomyocytes. The rate of contraction in the cells exposed to TC was compared with that in a control group where no TC was added to the culture medium.

For cultures of single cells, the percentage of contracting cardiomyocytes was calculated using separate groups of 25 individual cells. All measurements were made by a single observer. The proportion of cells that remained beating following exposure to TC was compared with that in a control group where no TC was added to the culture medium.

Investigation of the effects of TC on cardiomyocyte cell death
To establish whether cells were living or dead, fluorescein diacetate/propium bromide stain (Sigma, Poole, Dorset, U.K.) at a final concentration of 100 ng/ml was added to the cardiomyocyte cultures 3 min after TC administration [20]. The fluorescence of the cells was measured to determine whether cells were alive (indicated by a green colour) or dead (indicated by the nucleus staining red).

Calcium wave dynamics
The cardiomyocytes were loaded with the visible-wavelength fluo-3 Ca^{2+} indicator by cell incubation with the esterified derivative of fluo-3 (fluo-3 acetoxymethyl ester; Molecular Probes, Leiden, Netherlands) in a medium containing equal volumes of Leibovitz’s L-15 medium (Gibco) and Hanks balanced salt solution buffer (Gibco) at room temperature for 15 min. Then cells were re-washed five times with the medium, followed by a
tial period of 20 min to allow for complete intracellular dye cleavage [21,22].

Scanning laser confocal microscopy was used to study Ca$^{2+}$ dynamics in the cardiac myocyte network. Scanning was performed so that several elevations in Ca$^{2+}$ were visible in the adjacent cells in a single scan, allowing monitoring of cell synchronization and the rhythm of Ca$^{2+}$ wave propagation. We use a relatively unusual way of observing Ca$^{2+}$ transients. This is because we wanted to gain maximum information on cell rhythm and synchronization while introducing minimum laser irradiation to the sample, so we will expand on this method briefly. The low-power ($<0.05\, mW$) laser beam rapidly scanned the conglomerate of cells, chronologically, from the bottom up. In this case the Ca$^{2+}$ transients are recorded as bright horizontal bands. If the bands traverse all the cells in the field and the time distances between horizontal bands are equal, this indicates synchronous rhythmic beating of cells and adequate cell-to-cell conduction in the network (e.g. Figure 2A). The scanning laser confocal microscopy protocol has been described by Korchev et al. [23].

Statistics

Results are expressed as means±S.D. Comparisons were made using one-way ANOVA. The analysis was performed using the InStat statistics package (GraphPad Software).

RESULTS

Effects of TC on the rate of contraction and on the proportion of beating cardiomyocytes when cultured as single cells

Addition of TC to give a concentration of 0.3 mM in the culture medium of single cardiomyocytes caused a decrease in the mean rate of contraction after 1 h, from $68.7±5.7\, \text{beats/min (mean±S.D.)}$ to $31.4±5.3\, \text{beats/min (P < 0.001)}$. The rate had recovered to $58.0±8.6\, \text{beats/min}$ at 1 h after transfer to TC-free medium. A similar decrease in the rate of contraction was seen following the addition of TC to give a concentration of 3 mM, i.e. from $69.8±8.9$ to $25.4±7.6\, \text{beats/min (P < 0.001)}$, followed by recovery to $59.2±7.0\, \text{beats/min}$ (Figure 3A).

TC also affected the proportion of cells that demonstrated contractile activity. A concentration of 0.3 mM TC in the culture medium of single cardiomyocytes...
resulted in a decrease in the percentage of beating cells from 70.5% to 32.6% ($P < 0.001$). This returned to 59.2% following transfer of the cells to TC-free medium. A similar, but greater, decrease was seen with 3 mM TC, i.e. from 68.3% to 19.8% ($P < 0.001$), followed by recovery to 54.2% (Figure 3B).

**Figure 2** Effects of TC on calcium dynamics in cardiomyocytes

(A) Network of cardiomyocytes before the addition of TC to the culture medium. Horizontal lines represent calcium waves which extend across adjacent cells, consistent with synchronous beating. The spacing between the lines provides an indication of frequency. (B) The addition of 0.3 mM TC alters calcium wave dynamics, consistent with tachycardia (closely spaced lines; t), and with calcium overload with loss of contraction (o). The network of cells is no longer beating synchronously. (C) At 6 min after tachycardia, bradycardia (b) and overload (o) are seen. There is no synchronous contraction. Each scan took 20 s to acquire.

**Figure 3** Effects of TC on (A) rate of contraction and (B) the proportion of actively beating cardiomyocytes when cultured as single cells

Abbreviation: bpm, beats/min. Results are expressed as means and S.D. Comparisons were made using one-way ANOVA ($^*P < 0.001$).

**Effects of TC on the rate of contraction and integrity of networks of cardiomyocytes**

A concentration of 0.3 mM TC in the culture medium of networks of cardiomyocytes caused a similar decrease in the rate of contraction to that seen with single cells. These changes were not reversible after transfer to TC-free medium. The higher concentration (3 mM TC) resulted in destruction of the integrity of the network, and cells ceased to beat synchronously. Transfer to TC-free medium resulted in recovery of the integrity of the network, but cells had a reduced rate of contraction. These changes are summarized in Table 1.

**The effects of TC on cardiomyocytes do not cause cell death**

The addition of TC to the culture medium of cardiomyocytes when cultured either as single cells or as a network did not cause cell death. This was indicated by the reversibility of the cessation of beating that was seen in the majority of cardiomyocytes following transfer of cells to TC-free medium (Figure 3B). Cells that did not
recover contractile function were shown to be alive by the administration of fluorescein diacetate/propidium bromide stain. No red staining was observed, consistent with TC not causing cardiomyocyte death.

**Calcium wave experiments**

Addition of TC to single cells initially caused an increase in the frequency of Ca²⁺ waves. After approx. 6 min this was followed either by a decrease in wave frequency or by Ca²⁺ overload. These changes are consistent with a decreased frequency of Ca²⁺ transients, and were associated with a decrease in the rate of contraction and cessation of beating, as was observed 1 h after addition of TC (Figure 3).

When TC was added to the network of cells, the altered Ca²⁺ dynamics were different in adjacent cells, resulting in asynchronous beating. The intracellular Ca²⁺ dynamics of a 5-day-old culture of newborn-rat cardiomyocytes, after cell loading with fluo-3, are shown in Figure 2(A). Standard bright-field microscopy revealed synchronous rhythmic contraction of the network of cells, and this was confirmed by the synchronous Ca²⁺ transients throughout the cluster of cardiomyocytes. A concentration of 0.3 mM TC in the culture medium resulted in altered Ca²⁺ wave dynamics, with an increased Ca²⁺ transient rate, and with Ca²⁺ overload and loss of contraction (Figure 2B). The network of cells is no longer beating synchronously. At 6 min after the changes shown in Figure 2(B), bradycardia and overload were seen (Figure 2C). Thus a concentration of 0.3 mM TC in the culture medium resulted in altered intracellular Ca²⁺ dynamics and loss of synchronous contraction within this small cluster of cardiomyocytes. Similar changes were seen following the addition of TC to give a concentration of 3 mM.

**DISCUSSION**

We have demonstrated that the primary bile acid, TC, can alter the rate and rhythm of cardiomyocyte contraction and cause abnormal Ca²⁺ dynamics in this *in vitro* system. These data suggest that raised fetal serum bile acids in OC may result in the development of a fatal dysrhythmia and sudden intra-uterine death.

The mechanisms by which TC could cause a fatal fetal dysrhythmia in the intact heart include altered cardiomyocyte Ca²⁺ dynamics and impaired gap junction function, resulting in impaired propagation of conduction and subsequent loss of synchronous contraction. Taurine-conjugated bile acids have been shown to act as Ca²⁺ ionophores, causing a dose-dependent increase in the intracellular Ca²⁺ concentration [24,25]. This is a result of transient permeabilization of the endoplasmic reticulum to Ca²⁺. To date, this effect has only been demonstrated in hepatocytes, and was not seen in platelets or in a neuroblastoma cell line, neither of which have specific bile acid transporters in the cell membrane [25,26]. This is the first investigation of the effects of TC on Ca²⁺ concentration in cardiomyocytes. Increased Ca²⁺ cycling, coupled with the decrease in pH which would accompany the rapid beating [27], may also affect the function of intercellular gap junctions that provide low-resistance pathways for current flow between heart cells [18,28] and are thought to be crucial for beat synchronization [29,30] and for arrhythmia generation [20,21]. Intracellular calcium, through altered dynamics and overload, also has a pivotal role in the generation of arrhythmia [31,32].

Sudden cardiac death is usually attributed to tachyarrhythmias, although asystole and severe bradycardia are also distinct possibilities. In the latter two cases one would have to postulate substantial sino-atrial slowing, or partial to complete atrioventricular conduction block. We cannot, from our model, comment reliably on this type of conduction disturbance. We do see evidence of conduction block within the network. This cell-to-cell block could contribute to re-entry, an electrophysiological promoter of sustained tachyarrhythmias. Although our model is far from the clinical situation, our results would suggest ventricular tachycardia or fibrillation as the major contributors to cholestatically induced sudden death *in utero*.

Clinical studies of the fetal heart rate in OC pregnancies have demonstrated both tachycardia and bradycardia [1,8,12], consistent with the findings in the present study. However, intra-uterine death in OC is a sudden event, and fetal cardiotocography is not a reliable way of predicting the at-risk fetus [3,4]. This may be because there is a threshold above which the influence of bile

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**Table 1** Effects of TC on the rate of contraction of cardiomyocytes cultured as a network

<table>
<thead>
<tr>
<th>TC conc. (mM)</th>
<th>Before adding TC</th>
<th>1 h exposure to TC</th>
<th>1–2 h TC-free</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>102.9 ± 6.7</td>
<td>104 ± 7.2</td>
<td>102.5 ± 8.0</td>
</tr>
<tr>
<td>0.3</td>
<td>103.2 ± 13</td>
<td>55.3 ± 3.5*</td>
<td>54.7 ± 2.0*</td>
</tr>
<tr>
<td>3.0</td>
<td>105.3 ± 11.8</td>
<td>Li.</td>
<td>54.2 ± 5.5*</td>
</tr>
</tbody>
</table>

Values are means ± S.D. Significance of differences: *P < 0.001 compared with control. L.I., loss of integrity of the network.
acids, such as TC, is observed. In addition, there may be changes in cardiac function from 37 weeks’ gestation that result in an increased susceptibility of fetal cardiomyocytes to the effects of bile acids. This suggestion is supported by clinical studies that have demonstrated that delivery by 37–38 weeks’ gestation has reduced the perinatal mortality rate in OC from 10–15% [1,2] to 2.0–3.5% [2–4].

Severe bradycardia has been reported in neonates following exchange transfusion [33], and bile duct ligation in adult male rats can cause bradycardia, increases in PR and QT intervals, and arrhythmia in association with raised serum bilirubin levels, accumulation of bilirubin in the myocardium and depletion of cardiac glycogen [34]. However, there are no in vivo studies that demonstrate bradycardia in association with raised bile acids.

The concentration of 0.3 mM TC was chosen because this is within the range of concentrations of total bile acids that we have observed in the serum of women with OC (C. Williamson, unpublished work). A concentration of 3.0 mM was used for comparison. The concentration of TC or of other bile acids to which the fetal cardiomyocytes are exposed in vivo is difficult to quantify, and may be greater or smaller than the concentrations used in the present study.

We have investigated the effects of the same doses of TC on adult rat cardiomyocytes, and have found that adult myocytes are less susceptible to alterations in the rate of contraction than are neonatal myocytes (S. Harding, J. Gorelik, M. Lab, Y. Korchev and C. Williamson, unpublished work). This is consistent with the fact that mothers with OC have not been reported to develop dysrhythmias. This suggests that the fetal heart is more susceptible to the development of dysrhythmias in vivo. The normal fetal heart beats faster than the maternal heart, and this is consistent with more Ca\(^{2+}\) transients per unit time. Therefore a small increase in the Ca\(^{2+}\)-wave frequency could result in the breakdown of synchronized contraction via the cellular mechanisms alluded to above.

The advantage of the experimental model used in the present study is that it is possible to observe the effects of bile acids on single cells and on adjacent cells within the network. This is useful as a model of the cell-to-cell interactions that occur in the intact human heart. It will be of interest to extend the observations reported in the present study, in order to investigate the effects of bile acids on the intact fetal and maternal heart in animals and humans. An advantage of in vitro experiments such as those reported in this study is that they provide a better understanding of the mechanisms by which bile acids may impair cardiomyocyte function, prior to the commencement of in vivo experiments.

The pharmacological agents ursodeoxycholic acid and dexamethasone both improve maternal serum bile acid levels and the clinical features of OC [35–38], but it is currently not known whether their use improves fetal prognosis. Future experiments using this model will allow us to evaluate whether these agents may be of benefit to the fetus. We anticipate that subsequent studies in animals and humans may elucidate further the precise role of fetal cardiomyocyte damage in the unexplained late fetal death in OC.

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REFERENCES

10 Reference deleted
11 Reference deleted
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