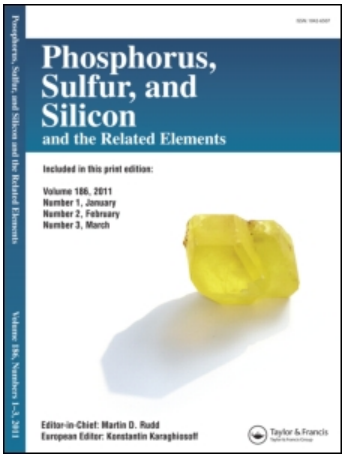

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^{31}P NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF CELLS AND TISSUES

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Abstract Phosphorus-31 magnetic resonance spectroscopy is an excellent technique for biological studies due mainly to its non-invasive nature. We describe some illustrative applications on phospholipid metabolism and hormone effects of breast cancer cells, and on skin metabolism and pharmacodynamics.

Key Words: Phosphorus-31 NMR, breast cancer, skin, pharmacodynamics, cells

INTRODUCTION

Phosphorus (^{31}P) is the most prominent nucleus studied by nuclear magnetic resonance (NMR) spectroscopy both *in vivo* and *in vitro*. ^{31}P NMR spectroscopy has been established as an excellent non-invasive procedure for studying the metabolism of some biological compounds of fundamental functional and structural importance, as well as a precise method to measure intracellular pH and membrane permeability.

In this article, we summarize recent work on the application of ^{31}P NMR spectroscopy to the metabolism of different cells and tissues in our laboratory, as examples of the multiple applications of this powerful technique. We will start with the results of our investigations to evaluate the biochemical status and physiological processes in perfused intact breast cancer cells, continuing with our *in vitro* measurements on epidermis with ^{31}P NMR spectroscopy as a basis for developing a mechanistically relevant topical corticosteroid bioequivalence technique.

^{31}P NMR SPECTROSCOPY OF BREAST CANCER CELL LINES

NMR studies of cellular metabolism can be performed with cellular extracts, cell suspensions, and perfused intact cells. Cellular perfusion is much preferred for NMR studies since metabolic processes can be continuously monitored (not possible with extracts), and cells can be studied for prolonged periods during perfusion under physiological conditions (very limited experiments are warranted with cell suspensions). There are now enough methods that an appropriate one can be adjusted to almost all cell types and experimental conditions. Comparisons and utilities of these methods have been recently reviewed[1].

During perfusion, substrates and nutrients are continuously furnished, and waste products removed, while stable pH levels and temperature of 37 °C are maintained. Perfusion is done with the cell's growth medium, at an appropriate flow rate, using a peristaltic pump. In ^{31}P NMR experiments, a phosphate-free medium is used in order not to interfere with intracellular pH determination, since its signal appears very close to the intracellular Pi resonance. A high density of cells ($2\text{-}3\times10^{8}$) must be present within the receiving coil since phosphorus NMR spectroscopy (or, generally, magnetic resonance) has low sensitivity. Thus it is difficult to perform a series of NMR studies of normal cell lines, e.g. with normal breast cells, since they grow very slowly in culture. In all experiments that are expected to last several days sterility must be maintained, and this should be considered in selecting the appropriate perfusion method. This can be accomplished by either using large capacity filters, or closed systems.

Two perfusion methods has been developed in our laboratory. The first method, which was introduced by Foxall and Cohen[2], is based on the properties of low-temperature gelling agarose. Cells are embedded in agarose threads prepared by extrusion of a cell suspension in a liquid gel through a cooled capillary tube. Since the cells are inside the matrix, the porosity of the matrix and the ease of nutrients diffusion are of critical importance. Penetration of molecules (smaller than albumin) has been determined. Attachment to the gel is not essential, so that both anchorage-dependent and -independent cells can be studied. However, due to the limited growth of anchorage-dependent cells in agarose threads, this method is not optimal for studies of cellular proliferation. The second method developed in our laboratory, based on a basement membrane (Matrigel), overcomes this obstacle.[3] Cancer cells grow in the Matrigel, and are morphologically identical to their *in vivo* counterparts. Most of our results have been obtained with agarose threads, although perfusion with cells embedded in Matrigel will establish a model tumor, most suitable for metabolic and pharmacological studies.

Initial experiments were performed to make peak assignments of the ^{31}P spectra. Cell extracts were used since better spectral resolution is obtained over intact cell threads allowing easier identification. Comparisons between normal and neoplastic breast cancer cells, and among different drug resistant breast cancer cell lines, have been done using ^{31}P NMR spectroscopy, with both extracts and agarose thread cell perfusion. These studies help delineate the differences in control of metabolic pathways between cells types.

Phospholipid metabolism has been extensively studied in our laboratory by ^{31}P NMR of perfused human breast cancer cell lines. Notable are the effects of substrates and inhibitors on specific enzymatic processes, such as ethanolamine kinase, which in the presence of extracellular ethanolamine resulted in the formation of PE (FIGURE 1). The effect of hemicholinium-3 in perfusate resulted in the reduction of the concentration of phosphocholine (PC). These studies showed that the second step in the process of phospholipid biosynthesis is rate limiting.

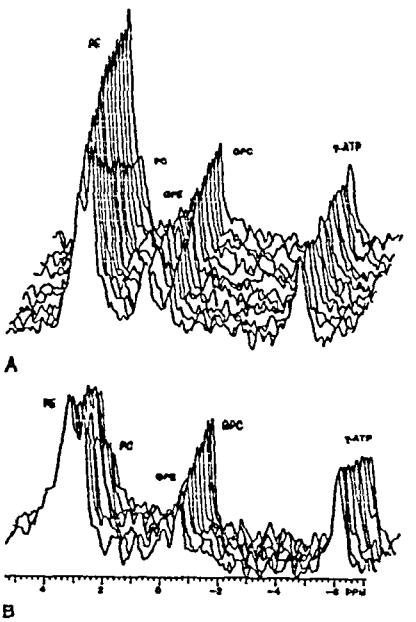

FIGURE 1 A. Effect of ethanolamine (2 mM) on the ^{31}P spectra of perfused breast cancer line (MDA-231) at 37 ^{o}C. B. Effect of hemicholinium-3, a specific inhibitor of choline kinase. Abbreviations are: phosphoethanolamine (PE), phosphocholine (PC), glycerophosphoethanolamine (GPE), and glycerophosphocoline (GPC).

Our efforts have also been directed to determine the effects of drugs and hormones on breast cancer cells. These results indicate that ^{31}P NMR can be used to measure the efficacy of an anti-neoplastic agent. A series of human breast cancer cells that vary in their estrogen and antiestrogen responsiveness was used to investigate their hormone growth dependence and the effects of tamoxifen. We observed no metabolic changes clearly associated with the metastatic phenotype. On the other hand, estrogen treatment produces no consistently significant changes in any of the cell lines, while a estrogen independent and estrogen responsive cell line responded to tamoxifen treatment by significantly increasing all spectral resonances (FIGURE 2).[4] This may reflect a tamoxifen-induced change to a more differentiated or apoptotic phenotype, or an attempt by the cells to reverse the inhibitory effects of the drug.

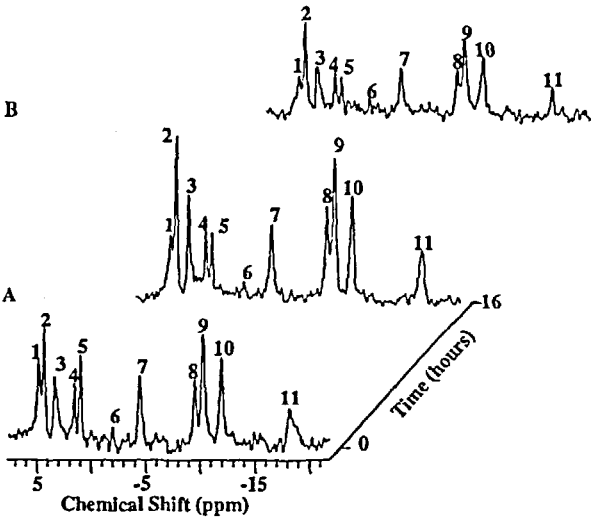

FIGURE 2. ^{31}P spectra of MIII breast cancer cells. A. baseline spectrum, and after 16 hr perfusion the cells were perfused for two hours with Tamoxifen (0.5 μM). B. Difference spectrum.

^{31}P NMR SPECTROSCOPY OF SKIN

We have recently measured the pharmacodynamic effects of dexamethasone on viable epidermis by following intracellular phosphate metabolism using ^{31}P NMR spectroscopy.[5] Changes to the concentrations of phosphate-containing metabolites in response to exposure to dexamethasone were an indication of the drug effects on the skin. In addition to demonstrating the applicability of determining pharmacodynamic relationships in epidermis for the modeling of drug effects in the skin during their absorption, this work can serve as a basis for developing a mechanistically relevant topical corticosteroid bioequivalence technique.

Strips of viable, enzymatically separated miniature swine epidermis were cut and placed into a 10 mm NMR tube and perfused with phosphate-free balanced salt solution during the experiment. The effect of the drug, dexamethasone, was measured on the epidermis by measuring changes in the concentrations of phosphate metabolites by NMR spectroscopy while exposing it to different drug concentrations. The concentrations of these metabolites remain constant in untreated epidermis for 18 or more hours of perfusion. The metabolites acting as sources of biochemical energy (NTPs and phosphocreatine (PCr)) showed consistent decreases in peak heights during drug perfusion with a return to baseline peak heights after 3 to 5 hr of washout. The decrease in PCr peak intensity was the change best correlated with drug dose. A log-linear dose-response relationship was observed (FIGURE 3).

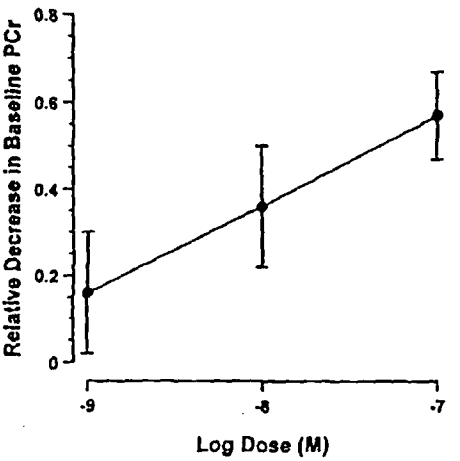

FIGURE 3. Log dose-response relationship for dexamethasone effect on reduction of epidermal phosphocreatine (PCr) levels

The data obtained from the NMR dose-response experiments was used to estimate parameters for an indirect model of pharmacodynamic effect. The model is based on the assumption that dexamethasone acts to reduce the production of PCr in the skin as described by the equation:

$$\frac{dPCr}{dt} = k_{in}\left(1 - \frac{C_{Epidermis}}{IC_{50} + C_{Epidermis}}\right) - k_{out}\ C_{PCr}$$

In this model, a basal rate of production of a PCr is expressed by the rate constant , k*in*, and a basal rate of elimination of PCr is expressed by the rate constant, k*out*. The drug effect is dependent on its concentration in the skin and the IC50. The IC50 is defined as the concentration of drug required to inhibit the production of PCr by 50%. The parameters, k*in*, k*out*, and IC50, were estimated by a simultaneous fit of the model to tissue concentrations of PCr, determined by the NMR spectroscopy dose-response experiments.

Dexamethasone exerts a reproducible and reversible effect upon phosphate metabolites within the epidermis. A dose-response relationship between dexamethasone and PCr was significant and reproducible. The correlation of dose of dexamethasone to PCr levels in epidermis could function as a surrogate pharmacodynamic endpoint for anti-inflammatory or antiproliferative action. The observation of an epidermal pharmacodynamic response by ^{31}P NMR spectroscopy and its incorporation into a pharmacodynamic model is an initial attempt at cellular level cutaneous pharmacodynamic modeling. Measurement of the dose-response relationship *in vitro* or *in vivo* through NMR techniques could function as a mechanistically relevant measure of topical corticosteroid bioequivalency.

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